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조선시대 미라 시료에서 추출한 선충류 및 흡충류 DNA에 대한 고병리학적 연구

Paleopathological study on nematode and trematode DNA extracted from specimens of Joseon Dynasty mummies

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Paleopathological study on nematode and trematode DNA extracted from specimens of Joseon Dynasty mummies

by
Jong Ha Hong

A thesis submitted to the Department of Anatomy in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Medicine at Seoul National University College of Medicine

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ABSTRACT

Introduction: Over the past several decades, ancient DNA (aDNA) study has been a significant area of interest in the fields of archeology, anthropology, and medicine. However, the number of aDNA cases reported so far has not been sufficient for obtainment of detailed information on parasites genetics. By paleoparasitological investigations in South Korea, I have collected a number of pre-modern Korean-mummy feces and tissue specimens containing parasite eggs. Utilizing those ancient specimens and their remnant eggs, I tried to analyze multiple DNA regions of *A. lumbricoides*, *T. trichiura*, *P. westermani*, *C. sinensis*, and *M. yokogawai* aDNA.

Methods: The samples utilized in this study were obtained from 15th to 18th century Joseon Dynasty mummies. Prior to the aDNA analyses, microscopic examination confirmed the presence of parasite eggs in each specimen. aDNAs from the parasite eggs were isolated and sequenced for multiple DNA regions. By BLAST search, I retrieved similar sequences at the National Center for Biotechnology Information (NCBI), and then inferred the phylogenetic relationships among the taxa by the Maximum

Likelihood (ML) method.

Results and Discussion: By agarose gel electrophoresis, the amplified products were detected in multiple DNA regions of five parasite species. Using the aligned clone sequences, consensus sequences were determined. The *Ascaris* and *Trichuris* sequences of the mummy specimens were highly similar to the sequences in GenBank thus far. The *P. westermani* CO1 and ITS2 sequences obtained from the Korean mummies showed genetic distinction from the other species of genus *Paragonimus*. Next, I showed that ITS1, NAD2, and NAD5 could be good markers for molecular diagnosis of *C. sinensis*. Finally, the *M. yokogawai* aDNA (CO1) sequence was distinctly clustered relative to those of *M. takahashii* and *M. miyatai*. I found that each parasite DNA sequence belonged to separate clusters that were evidently distinct from those of the other parasite species reported to date.

Conclusion: In this study, I successfully analyzed the aDNA sequences of five different parasite species. Considering that there have been very few or no reports on parasite aDNA so far, the current study could expand the existing gene pool of parasite paleogenetics. Even so, I also admit that aDNA investigations of much wider geo-historical scope are still required in order to uncover the exact evolutionary histories of these

parasites.

Keyword: Parasite; *Ascaris lumbricoides*; *Trichuris trichiura*; *Paragonimus westermani*; *Clonorchis sinensis*; *Metagonimus yokogawai*; Ancient DNA

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Introduction

Higuchi et al. (1984) and Pääbo (1985) successfully analyzed ancient DNA (aDNA) retrieved from human and animal bones collected at archeological excavation sites. Over the course of the succeeding several decades, aDNA study has become a significant area of interest in the fields of archeology, anthropology, and medicine (Marota and Rollo, 2002; Cipollaro et al., 2005; Geigl and Grange, 2018).

In particular, scholars have achieved remarkable outcomes in studies on ancient infectious diseases. Maixner et al. (2016) restored 5,300-year-old *Helicobacter pylori* aDNA from the European Copper Age glacier mummy. aDNA studies have been conducted to verify that *Yersinia pestis* and *Vivrio cholera* were popular epidemics in Europe. Bos et al. (2011) reported an ancient genome of *Yersinia pestis* from Black Death victims dated to instances of pestilence-associated mortality in medieval England. Devault et al. (2014) extracted and analyzed *Vivrio cholera* aDNA from a patient who had died in the 1849 Philadelphia outbreak.

Studies on ancient pathogens have been carried out in Korea as well. Kahila Bar-Gal et al. (2012) successfully analyzed Hepatitis B virus DNA from a 16th century child mummy excavated

at a Korean archaeological site. A similar study on ancient pathogens was conducted by Shin et al. (2018), who successfully identified vacA (s- and m-region) alleles of *Helicobacter pylori* aDNA from two 17th century Korean-mummy specimens.

Meanwhile, there have also been attempts to analyze the DNA of parasite species found at archaeological sites. For the first time ever, Loreille et al. (2001) recovered *Ascaris lumbricoides* and *Ascaris summ* aDNA from coprolites unearthed at a 14th century archaeological site in Belgium (Loreille et al., 2001; Iñiguez, 2014). Ancient *Ascaris* DNA sequences of 18S rRNA and cytochrome B (cyt b) also have been identified from specimens obtained from the 15th to 18th century Seocheon and SN2-19-1 mummies (Oh et al., 2010, 2015).

In the case of *Trichuris trichiura*, the earliest aDNA analysis concerned an SSU rRNA sequence retrieved from mid-18th century human remains in South Korea (Oh et al., 2010). Myšková et al. (2014) amplified the *Trichuris* SSU rRNA gene using 18th-19th century soil sediments collected at an excavation site in the Czech Republic. Søe et al. (2015, 2018) reported *T. trichiura* mitochondrial DNA (mtDNA) sequences isolated from 5th to 18th century soil sediments from Denmark, Bahrain, the Netherlands, and Lithuania.

As for *Paragonimus*, *Clonorchis*, and *Metagonimus* spp., however, very little paleogenetic data has been reported so far. Heretofore, the *P. westermani* ITS2 aDNA sequence was acquired from a 17th century Korean mummy (Shin et al., 2012). In the case of *Clonorchis sinensis,* Shin et al. (2013) analyzed aDNA sequences using fecal specimens from a 17th century Korean mummy.

Recently, parasitologists have attempted, by aDNA analyses, to derive conclusions on comprehensive topics such as the diagnosis of existing parasites, regional differences among various parasite genome sequences, and the migration routes of ancient populations. For example, Hawash et al. (2015) found a regional difference between *T. trichiura* whole-mitochondrial genome sequences from China and Uganda. *P. westermani*, moreover, is well known to have been clustered into two or more groups by DNA phylogenetic analyses (Iwagami et al., 2003; Binchai et al., 2007; Doanh et al., 2009; Devi et al., 2013; Blair et al., 2016).

These findings suggest that obtaining much more detailed understandings of parasites' genetic histories will require retrieval of additional aDNA sequences, not only from modern parasite samples but also from archaeological samples representing a wider geo-historical scope. However, despite such pioneering studies' uncovering of the genetic traits of several parasites, the number of

aDNA cases reported to date is insufficient for any detailed understanding of parasite genetics.

Fortunately, by paleoparasitological studies in South Korea over the past several years, I have been able to collect a number of pre-modern Korean-mummy fecal or tissue specimens in which the presence of parasite eggs was microscopically confirmed (Seo et al., 2014, 2017). Utilizing those ancient specimens, in this study, I tried to analyze multiple DNA regions of *A. lumbricoides, T. trichiura, P. westermani, C. sinensis*, and *M. yokogawai* aDNA.

The current report on these five species of parasite aDNA was thus designed to uncover genetic information on parasites prevalent among pre-20th century Korean people. I endeavored not only to reveal the DNA sequences of parasites that had infected people of the Joseon Dynasty but also to elucidate, by phylogenetic analysis, their genetic characteristics. The data thus obtained could expand the spatio-temporal scope of parasitological research on the genetic history of parasites.

CHAPTER 1

Ascaris DNA sequences of cytochrome b, cytochrome c oxidase subunit 1, NADH dehydrogenase subunit 1, and internal transcribed spacer 1 DNA regions from Joseon mummy feces

Introduction

As a means of revealing secular changes in the genetic traits of *Ascaris* spp., aDNA analysis has become an indispensable tool of paleoparasitology. In previous studies, aDNA analyses were performed on *Ascaris* eggs obtained from 15th– to 18th–century Korean mummies. Briefly, from Seocheon and SN2–19–1 mummies, *Ascaris* 18S rRNA and cytochrome b (cyt b) mitochondrial DNA regions were successfully extracted and sequenced (Oh et al., 2010, 2015). These outcomes are fundamental to the understanding of the genetic characteristics of the *Ascaris* spp. that infected pre–modern Korean peoples.

The *Ascaris* aDNA data pool obtained thus far is nonetheless insufficient for the reconstruction of the complete genetic history of those species. In the 2 previous reports mentioned above, only 1 and 2 *Ascaris* DNA regions from single ancient feces samples were analyzed (Oh et al., 2010, 2015). Multiple *Ascaris* DNA regions from multiple ancient feces samples obtained from as many archaeological sites as possible are still needed for more advanced analyses.

Fortunately, over the course of the past several years, I have been able to collect additional and larger numbers of ancient

mummy fecal and precipitate samples for which microscopic examination has confirmed the presence of *Ascaris* eggs (Seo et al., 2017). Utilizing these samples in the present study, I analyzed *Ascaris* cytochrome b, cytochrome c oxidase subunit 1 (CO1), NADH dehydrogenase subunit 1 (NAD1), and internal transcribed spacer 1 (ITS1) DNA regions. *Ascaris* CO1 and NAD1 were included in this study, owing to the impressive results earlier obtained using archaeological samples (Peng et al., 2005; Søe et al., 2015). In the case of the *Ascaris* ITS1, it had already been established as an effective genetic marker for characterization of global *Ascaris* populations (Das et al., 2015). Interestingly, there have been no reports of any recovery of the *Ascaris* ITS1 from archaeological samples, which fact may be considered to amplify the significance of the present examination of Joseon-period samples.

Materials and Methods

The ancient feces and precipitates used in this study were obtained from 15th– to 18th–century Joseon mummies (n=8; Junggye, Dalsung, YG2–4, YG2–6, Hwasung, Jinju, Seocheon, and Cheongdo). The archaeological information about each case is summarized in Table 1. The ancient feces were sampled from the intestines of mummies (Dalsung, Hwasung, Jinju, and Cheongdo) by autopsy. Precipitates on hip bones in semi–mummification cases (Junggye, YG2–4, YG2–6, and Seocheon) were also sampled.

The samples could be collected only after complete removal of multiple layers of clothing. Unauthorized contact with the samples was strictly prevented in this study. The probability of sample contamination from the outside was thus reduced as much as possible.

For each sample, the presence of ancient *Ascaris* eggs was determined by microscopic observation. Briefly, the sample was rehydrated with 0.5% trisodium phosphate solution for 1 week (Han et al., 2003; Reinhard and Urban, 2003). After the samples in–solution were filtered through multilayered gauze, they were precipitated for a day. When the precipitates were dissolved again in 0.5% trisodium phosphate solution (final volume 20 ml), the

solution was dropped onto the slides. A total of 200 μl of each sample was examined by light microscopy. Some of the microscopic data were already reported in our earlier studies (Oh et al., 2010; Seo et al., 2017). Subsequent aDNA analysis was performed on egg-positive samples. Our aDNA lab facility was set up in accordance with Hofreiter et al. (2001) and Willerslev and Cooper's (2005) Criteria of Authentication. At every stage of the experimentation, the researchers wore protection gloves, masks, gowns, and head caps.

The samples were treated with 1 ml lysis buffer (EDTA 50 mM, pH 8.0; 1 mg/ml of proteinase K; SDS 1%; 0.1 M DTT) at 56℃ for 24 hr. aDNA was extracted by phenol/chloroform/isoamyl alcohol (25:24:1). Primers for the *Ascaris* cyt b, CO1, NAD1, and ITS1 DNA regions had been generated at Integrated DNA Technologies, Inc. (Iowa City, Iowa). The pertinent primer information is summarized in Table 2.

DNA amplification was performed with a 25 μl reaction mixture containing AmpliTaq Gold 360 master mix (Applied Biosystems, Foster City, California) and 10 pmol of each primer. The polymerase chain reaction (PCR) conditions were as follows: pre-denaturation at 94℃ for 10 min, 50 cycles of denaturation at 94℃ for 45 sec, annealing at 52–65℃ for 45 sec, extension at 72℃

for 45 sec, and final extension at 72°C for 10 min. The PCR products were separated on 2.5% agarose gel (Invitrogen, Carlsbad, California) and then stained with ethidium bromide. Negative controls (extraction controls) were subjected to electrophoresis at the same time. They were photographed using a Vilber Lourmat ETX-20M equipped with Biocapt software (Vilber Lourmat, Collégien, France).

Cloning and sequencing was performed for the amplified PCR products of the expected sizes. Briefly summarized, the aDNA in an amplified band was extracted using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Bacterial transformation with amplified DNA product was performed using the pGEM-T Easy Vector System (Promega, Madison, Wisconsin).

Transformed bacteria were then grown for the next 14 hr in agar plates containing ampicillin (100 μg/ml), 0.5 mM IPTG, and XGAL (40 μg/ml). After selected colonies were grown once again in LB media for 12 hr, plasmids were harvested using the QIAprep Spin Miniprep Kit (Qiagen). Sequencing was performed on each strand using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) in the 3730xl Automated Sequencer (Applied Biosystems).

To identify the consensus sequences, cloning and

sequencing were repeated for each specific amplicon. Through these trials, 3–5 clone sequences were successfully obtained from each amplicon. Multiple sequence alignment was performed for each gene by Clustal W as implemented in MEGA6 (Tamura et al., 2013). Using the aligned clone sequences, consensus sequences of *Ascaris* genes were then determined for each mummy case.

The obtained consensus sequences were then compared to those available in GenBank using the National Center for Biotechnology Information (NCBI)/BLAST tools (Altschul et al., 1997). The Web Browser module and Alignment Explorer in MEGA6 were used to retrieve sequences homologous to those of interest from GenBank. Every retrieved sequence was deemed to be of the family Ascarididae. *Toxocara*, *Toxascaris*, and *Baylisascaris* also were chosen for comparison.

To estimate the evolutionary divergences among the *Ascaris* cyt b, CO1, NAD1, and ITS1 gene sequences, the respective numbers of base substitutions per site were counted. Analyses were performed using the Maximum Composite Likelihood model (Tamura et al., 2004). Pairwise distances were then calculated among the taxon sequences.

The evolutionary trees of *Ascaris* cyt b, CO1, NAD1, and ITS1 were inferred by the Maximum Likelihood (ML) method

(Saitou and Nei, 1987) implemented in the MEGA6 program (Tamura et al., 2013). The selected parameters were as follows: the Hasegawa-Kishino-Yuno model (cyt b, CO1, and NAD1) or the Kimura 2-parameter model (ITS1) for Model/Method; Uniform Rates (cyt b) or Gamma Distribution (CO1, NAD1, and ITS1) for Rates among Sites; complete deletion for gaps/missing data treatment and Nearest-Neighbor-Interchange (NNI) for the ML Heuristic Method. To confirm the reliability of the tree, MEGA6's bootstrap test was run for 500 bootstrap replicates (Felsenstein, 1985; Hall, 2011).

In light of the low bootstrap values presented in these trees, I constructed trees out of concatenated alignments of the *Ascaris* mitochondrial DNA regions (Thiergart et al., 2014). Following the method of Devulder et al. (2005), a DNA sequence of 840 base pairs was made by concatenation of the cyt b, CO1, and NAD1 sequences obtained in this study. Using the MEGA6 program, a phylogenetic tree was then generated for the concatenated DNA regions fragments. The selected parameters were the Hasegawa-Kishino-Yuno model for Model/Method, Gamma Distribution for Rates among Sites, complete deletion for gaps/missing data treatment, and NNI for the ML Heuristic Method. The number of bootstrap replicates was 1,000 (Felsenstein, 1985, Hall, 2011).

Table 1. The archaeological information of Korean mummies in this study.

Cases	Estimated Date	Sample Type	sex	Year of Discovery
Junggye	16C-17C	Precipitates upon hipbones	M	2014
Dalsung	16C-17C	Feces	F	2014
YG2-4	15C-16C	Precipitates upon hipbones	F	2012
YG2-6	15C-16C	Precipitates upon hipbones	F	2012
Hwasung	18C	Feces	M	2012
Jinju	16-17C	Feces	M	2010
Seocheon	17C	Feces	F	2008
Cheongdo	17C	Feces	M	2014

Table 2. List of primers used for amplification of *Ascaris* spp. DNA in this study.

Locus	Primers	Sequence (5' to 3')	Annealing Temperature	Amplicon Size (bp)
cyt b	Asc1	GTT AGG TTA CCG TCT AGT AAG G	52	142
	Asc2	CAC TCA AAA AGG CCA AAG CAC C		
CO1	As-Co1F	TTT TTT GGT CAT CCT GAG GTT TAT	55-59	146
	ACR1B	CAA CAC AAC CAA TCA AAC CAA		
	ACF2	TGG GTC TTT GGG TAT GGT TT	55	167
	ACR2	ACC AAA CAA GGT AGC CAA CC		
	ACF3B	TGG TTA TTG CTG TTC CTA CTG G	55-59	210
	As-Co1R	ACA TAA TGA AAA TGA CTA ACA AC		
NAD1	MH5F	TAT GAG CGT CAT TTA TTG GG	58	161
	NAD R1	CCA GGA ACC AAA ATA AAA GAA		
	NAD F2	TCC TTT GAA TTC TTC GGA AA	56	190
	NAD R2	AGA CTT TCT TAC CAT ACC ACT A		
	NAD F3	TGT GTT TGA TTG GGT TTT C	58	150
	As-NDR	GCA TCA CAA TAG CCA ACA AAT AC		
ITS1	ITS1 F1	CGA GCA GAA AAA AAA AAG TCT CC	63	187
	ITS1 R1	AAG CCC AAC ATG CCA CCT ATT C		
	ITS1 F2	TTT GGC CGA CAA TTG CAT GCG A	60	167
	ITS1 R2	CTA CCG ACG GTC CAG GCG ATA G		
	ITS1 F3	CCG CTA TTT CGT AAC AAC GGT	58	137
	ITS1 R3	CCT CCA AGC TGA GGC TCA TTG		
	ITS1 F4	AAA GCT CCT CGT TTC GAG TCG	56	212
	ITS1 R4	CGC ACT ATT TAT CGC AGC TAG		

Results and Discussion

By agarose gel electrophoresis, the amplified products of the *Ascaris* cyt b, CO1, NAD1, and ITS1 were detected in multiple samples, whereas the negative controls (extraction controls) did not exhibit any amplified bands (Figure 1). Cyt b-specific bands were found in the Junggye, Seocheon, YG2-4, Cheongdo, and Dalsung samples. CO1- and NAD1-specific bands were identified in the Junggye, Seocheon, YG2-4, and Cheongdo samples. The ITS1 showed specific bands only in the YG2-4 and Cheongdo samples. Using the aligned clone sequences, consensus sequences of *Ascaris* genes were then determined for each mummy case (5 cases for cyt b; 4 cases for CO1; 4 cases for NAD1; 2 cases for ITS1) (Figure 2).

In the results, the ancient Joseon cyt b, CO1, NAD1, and ITS1 DNA regions were highly similar to those of *A. lumbricoides* and *A. suum* reported here but were genetically distant from the *Toxascaris leonine*, *Baylisascaris procyonis*, and *Baylisascaris transfuga* (Figure 2). The cyt b sequences of the Joseon *Ascaris* spp. exhibited complete matching to each other. However, it not every aDNA sequence of each *Ascaris* showed 100% matching between them one another. Briefly, the CO1 sequences of YG2-4

and Cheongdo were different from those of Junggye and Seocheon *Ascaris*. A similar pattern was observed for NAD1. Furthermore, the Junggye and YG2–4 NAD1 sequences were not 100% identical to those of Seocheon and Cheongdo. Finally, the ITS1 sequences of YG2–4 and Cheongdo also were different from each other (Figure 3). In summary, I determined that the genetic characteristics of *Ascaris* prevalent in Joseon period Korea were not uniform but were diverse to some degree.

To estimate the evolutionary divergences among the *Ascaris* cyt b, CO1, NAD1, and ITS1 sequences, the respective numbers of base substitutions per site were counted. Pairwise distances were then calculated among the taxon sequences. The results are summarized in Figure 4.

The phylogenetic ML trees of the *Ascaris* cyt b, CO1, NAD1, and ITS1 with their bootstrap values are summarized in Figure 5. Every sequence of *Ascaris* spp. obtained from the Joseon samples was separately clustered from those of the *Baylisascaris*, *Parascaris*, *Hysterothylacium*, and *Toxascaris* spp. (Figure 5A–5D). As *A. lumbricoides* and *A. suum* did not make separate clusters in any of the genes' phylogenetic trees (Figure 5A–5D), we could reaffirm the existing claim that their differentiation is very difficult with any genetic markers (Leles et al., 2012; Liu et al., 2012; Shao

et al., 2014). As evidenced by the indicated increases in the bootstrap value (Figure 5E), the concatenation contributed to a considerable enhancement of tree robustness. In the ML tree of the concatenated aDNA sequences, *Ascaris* spp. was not grouped with the *Baylisascaris*, *Parascaris*, or *Toxascaris* spp. (Figure 5E). My result also confirmed that genetic characteristics of *Ascaris* of the Joseon period were not uniform. In terms of phylogenetic outcomes, this study re-confirmed that *Ascaris* genes have complicated aspects in the differentiation between *A. lumbicoides* and *A. suum*.

The genetic study of *Ascaris* aDNA is of great interest to parasitologists. Fuller and much more detailed understanding of the genetic history of *Ascaris* spp. will require the retrieval of additional *Ascaris* aDNA sequences, not only from modern samples but also from archaeological samples representing a significantly wider geo-historical scope. The present aDNA data on *Ascaris* eggs recovered from 15th- to 18th-century Joseon samples represent a meaningful step in that direction.

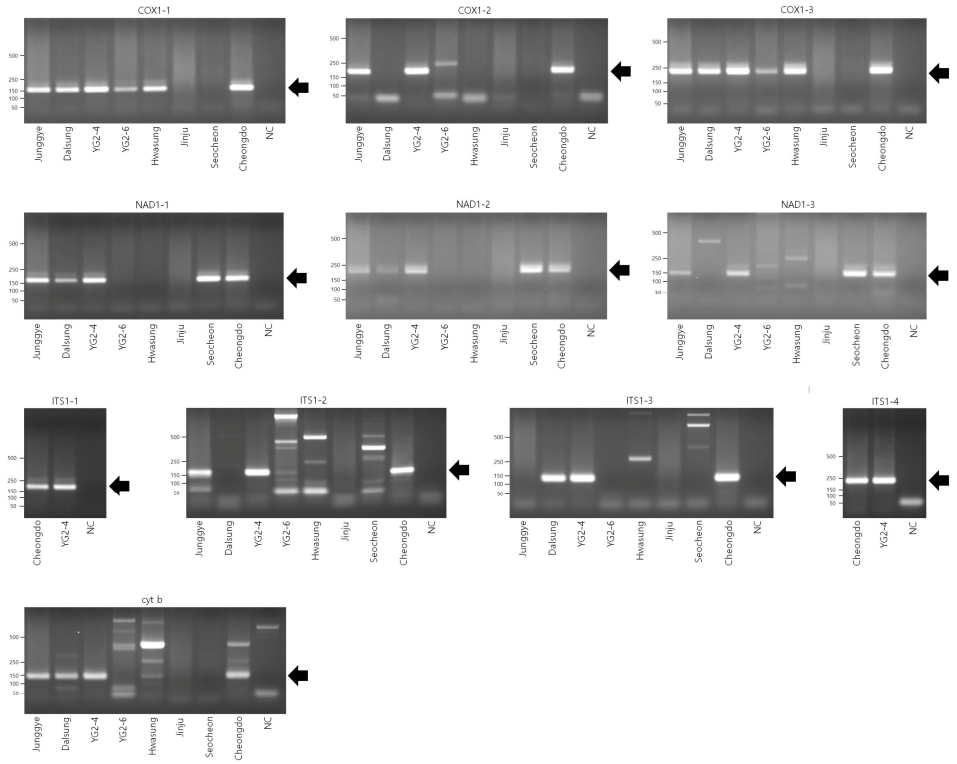

Figure 1. Agarose gel electrophoresis of *Ascaris* cyt b (142bp); fragments 1 (146bp), 2 (167bp), 3 (210bp) of CO1; fragments 1 (161bp), 2 (190bp), and 3 (150bp) of NAD1; and fragments 1 (187bp), 2 (167bp), 3 (137bp), and 4 (212bp) of ITS1 regions. The samples are Junggye, Dalsung, YG2−4, YG2−6, Hwasung, Jinju, Seocheon, and Cheongdo mummy feces. Arrows indicate each specific amplicon.

Ascaris cytb: Junggye mummy

```
          11018                                                                                                  11115
Consensus TTTTGACTTATGGTTGGAAttttggtagtatgttgggtatggttttaggttttcagattttgactggtacttttttggctttttATTATTCTAATGAT
Clone 1   .......................................................................................................
Clone 2   .......................................................................................................
Clone 3   .......................................................................................................
Clone 4   .......................................................................................................
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Ascaris cytb: Seocheon mummy

```
          11018                                                                                                  11115
Consensus TTTTGACTTATGGTTGGAAttttggtagtatgttgggtatggttttaggttttcagattttgactggtacttttttggctttttATTATTCTAATGAT
Clone 1   .......................................................................................................
Clone 2   .......................................................................................................
Clone 3   ....A..................................................................................................
Clone 4   .......................................................................................................
Clone 5   .......................................................................................................
```

Ascaris cytb: YG2-4 mummy

```
          11018                                                                                                  11115
Consensus TTTTGACTTATGGTTGGAAttttggtagtatgttgggtatggttttaggttttcagattttgactggtacttttttggctttttATTATTCTAATGAT
Clone 1   .......................................................................................................
Clone 2   .......................................................................................................
Clone 3   .......................................................................................................
Clone 4   .......................................................................................................
Clone 5   .......................................................................................................
```

Ascaris cytb: Cheongdo mummy

```
          11018                                                                                                  11115
Consensus TTTTGACTTATGGTTGGAAttttggtagtatgttgggtatggttttaggttttcagattttgactggtacttttttggctttttATTATTCTAATGAT
Clone 1   .......................................................................................................
Clone 2   ..........................................................G..........................................
Clone 3   .......................................................................................................
Clone 4   .......................................................................................................
Clone 5   .......................................................................................................
```

Ascaris cytb: Dalsung mummy

```
          11018                                                                                                  11115
Consensus TTTTGACTTATGGTTGGAATTTTGGTAGTATGTTGGGTATGGTTTTAGGTTTTCAGATTTTGACTGGTACTTTTTTGGCTTTTTATTATTCTAATGAT
Clone 1   .......................................................................................................
Clone 2   .......................................................................................................
Clone 3   .......................................................................................................
```

Figure 2-1. Aligned clone sequences of cyt b fragments from Junggye, Seocheon, YG2-4, Cheongdo, and Dalsung mummies.

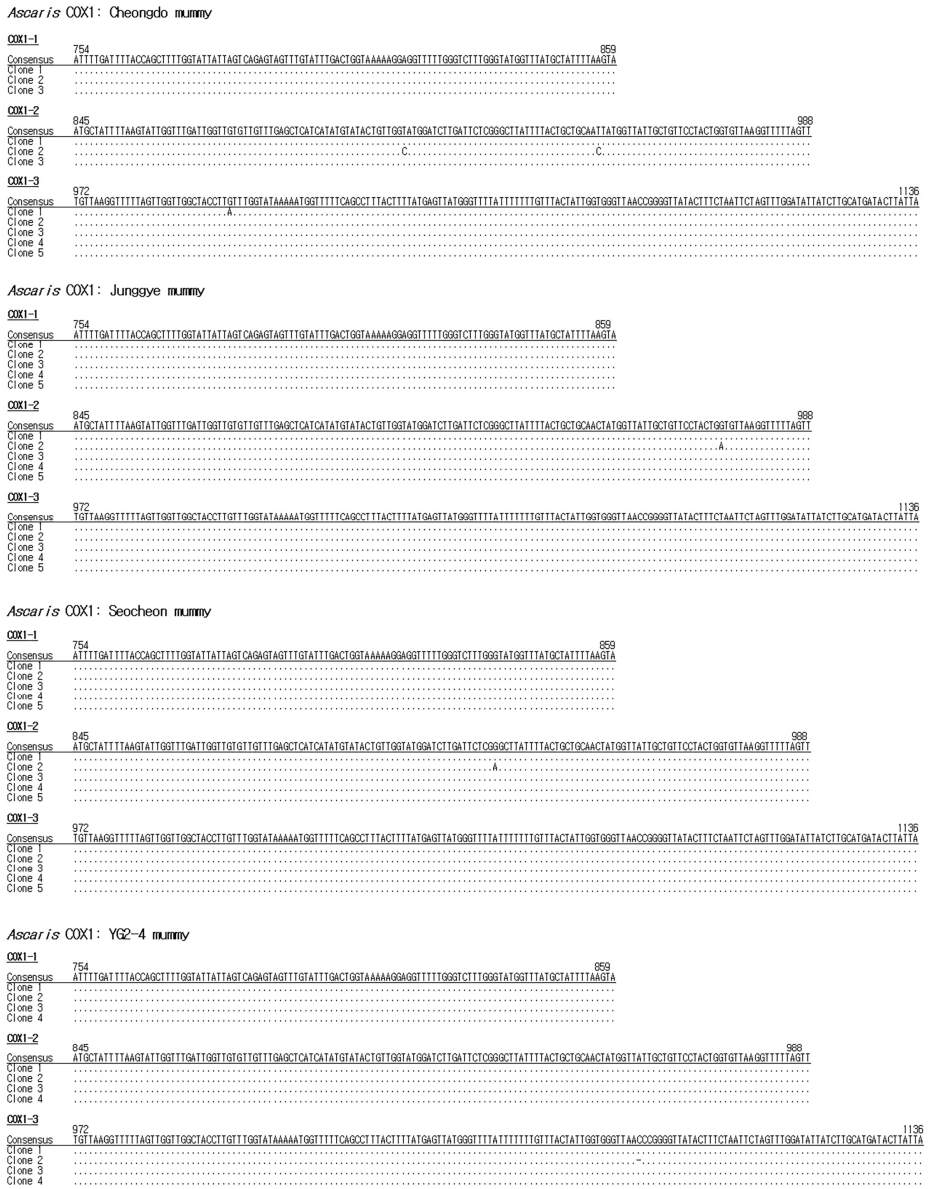

Figure 2-2. Aligned clone sequences of CO1 fragments from Cheongdo, Junggye, Seocheon, and YG2-4 mummies.

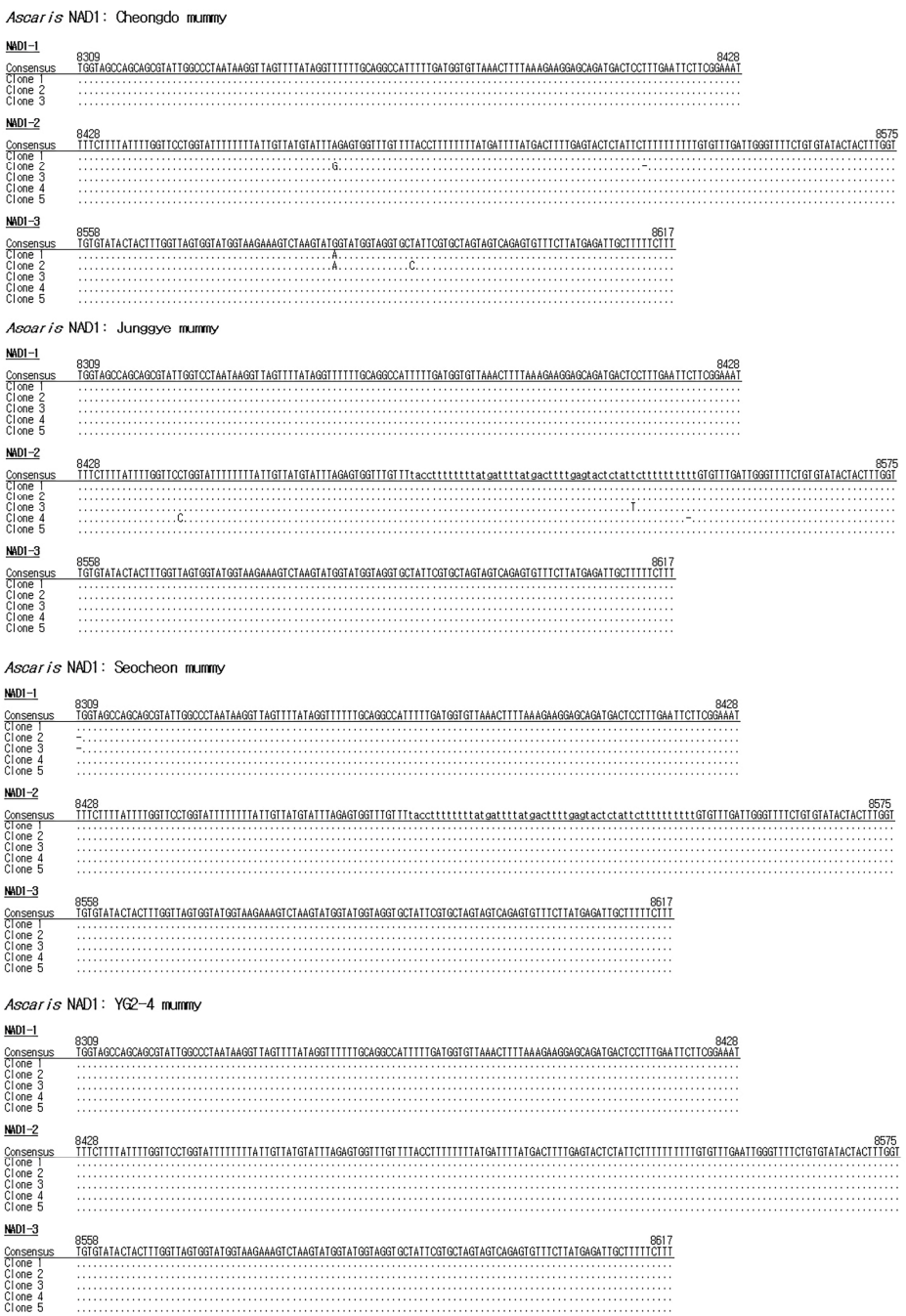

Figure 2-3. Aligned clone sequences of NAD1 fragments from Cheongdo, Junggye, Seocheon, and YG2-4 mummies.

Figure 2-4. Aligned clone sequences of ITS1 fragments from Cheongdo and YG2-4 mummies.

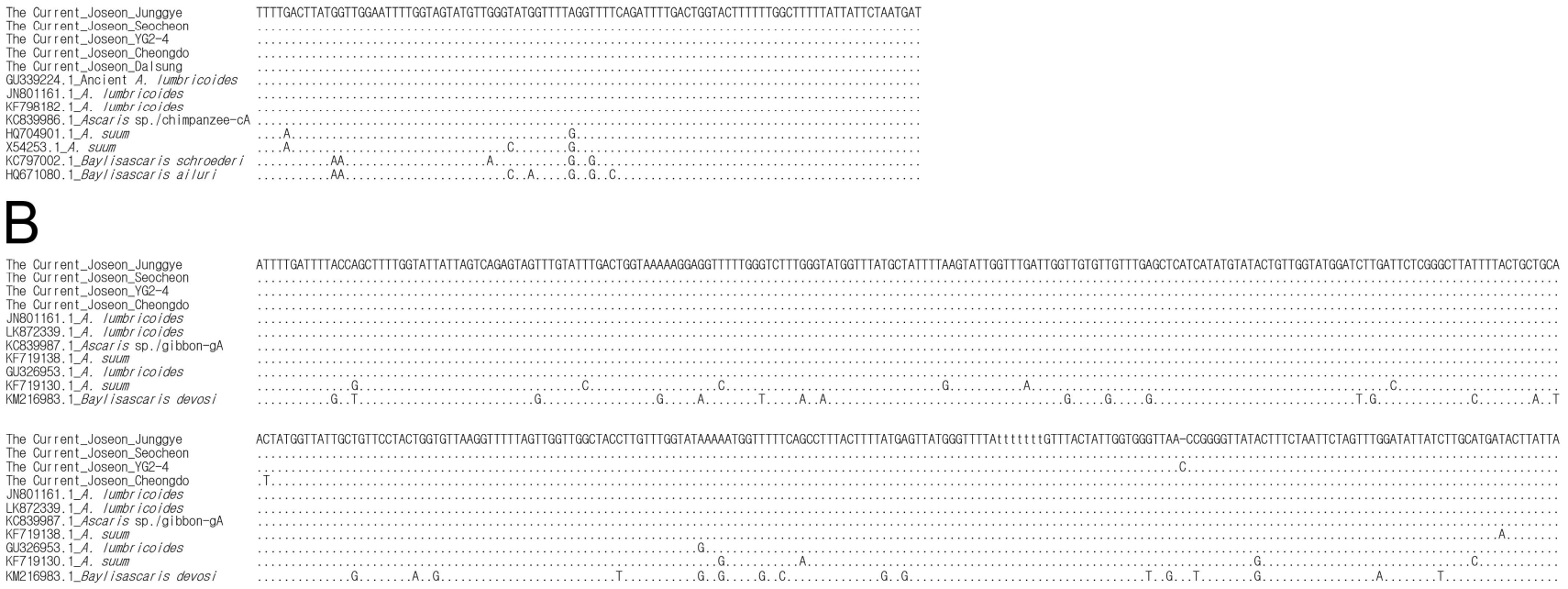

Figure 3-1. Comparison of consensus sequence of *Ascaris* (A) cyt b and (B) CO1 sequences from 15th- to 18th-century Korean mummy feces to those available in GenBank. The current cyt b sequences in (A) were identical to each other and were also the same as identified in previous report from the Seocheon mummy (GU339224.1).

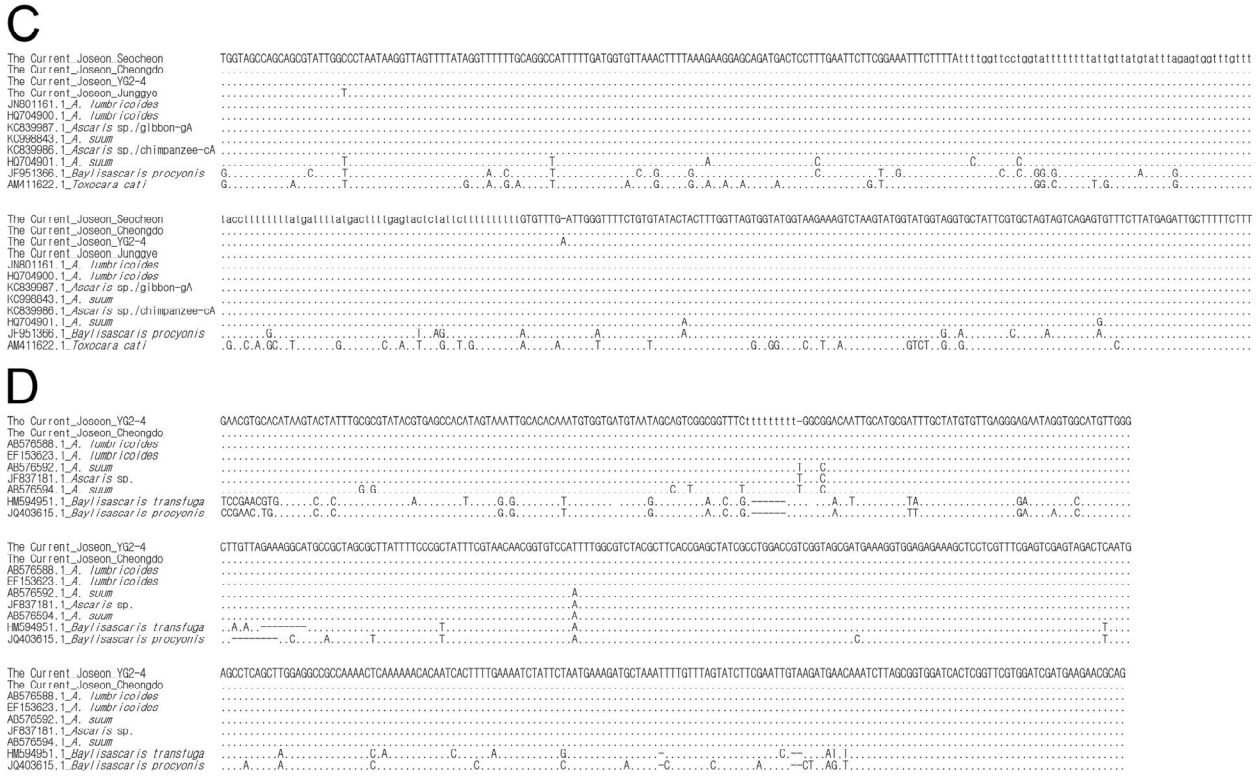

Figure 3-2. Comparison of consensus sequence of *Ascaris* (C) NAD1 and (D) ITS1 gene sequences from 15th- to 18th-century Korean mummy feces to those available in GenBank.

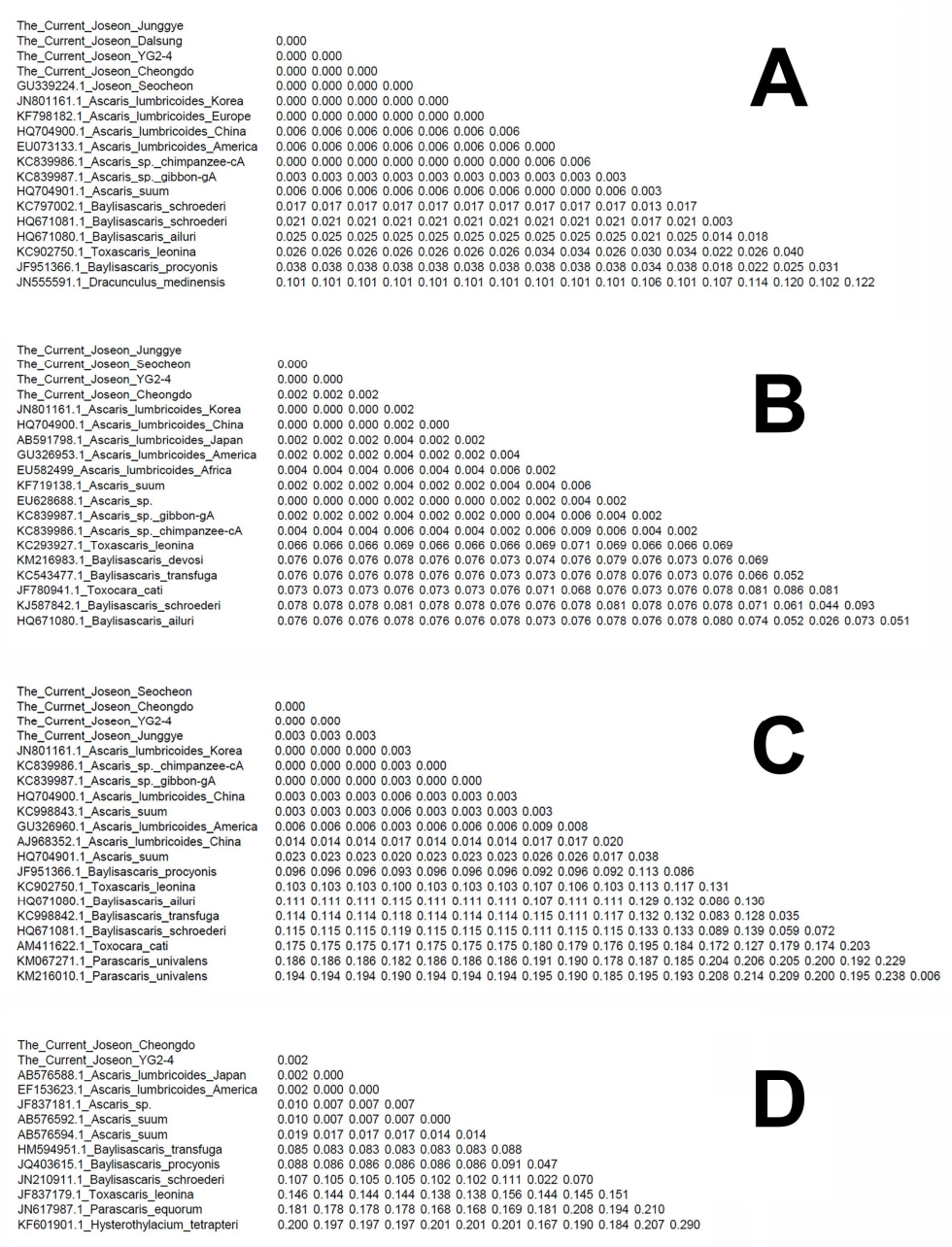

Figure 4. The pairwise distance of *Ascaris* DNA regions: (A) cyt b, (B) CO1, (C) NAD1, and (D) ITS1.

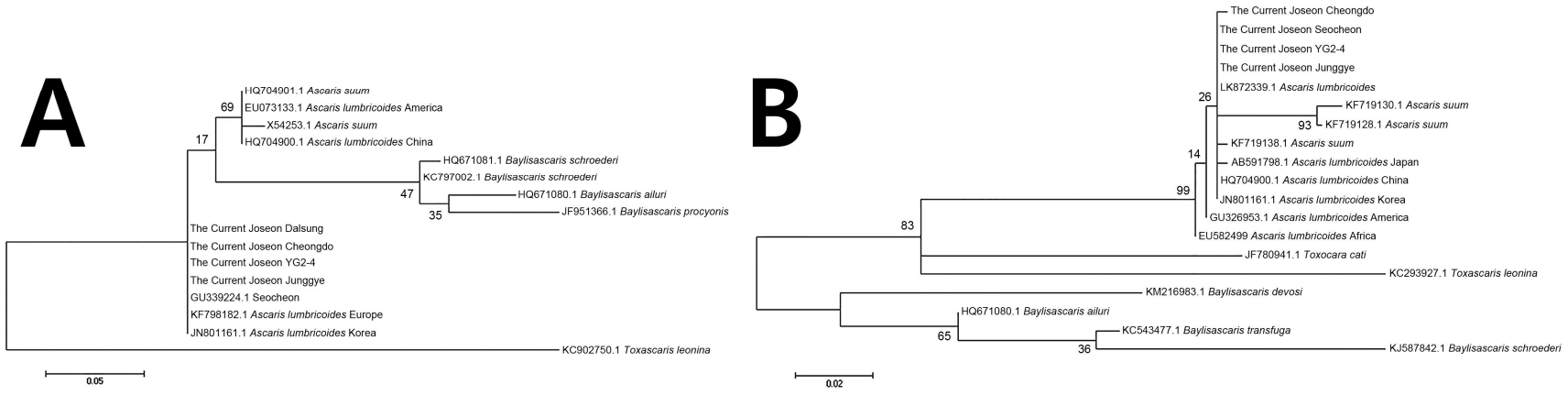

Figure 5-1. Maximum likelihood tree of *Ascaris* (A) cyt b and (B) CO1 sequences of mitochondrial DNA regions. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are marked next to the branches.

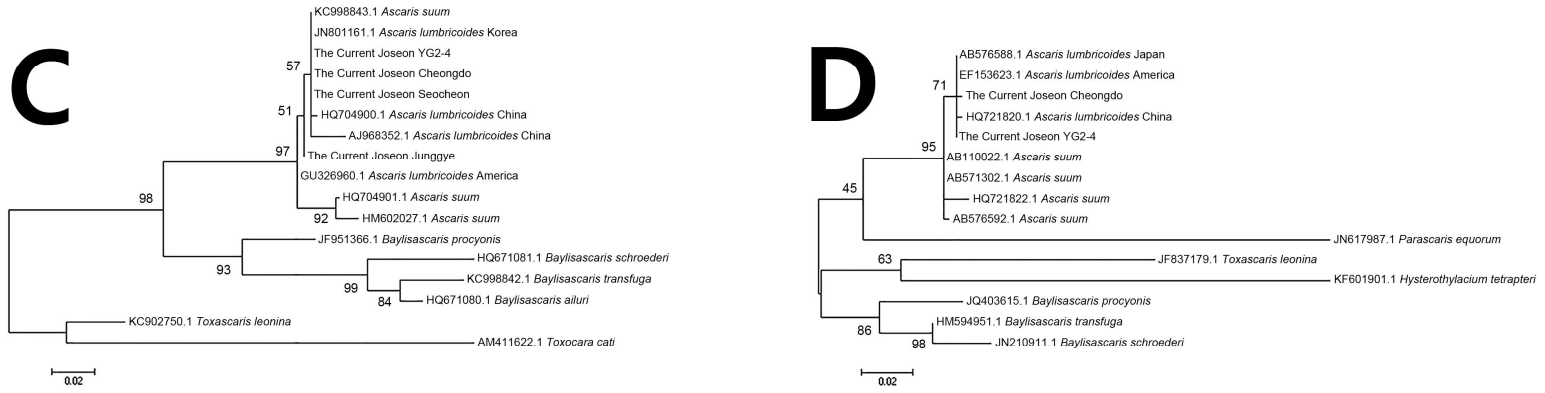

Figure 5-2. Maximum likelihood tree of *Ascaris* (C) NAD1 and (D) ITS1 sequences of mitochondrial DNA regions. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are marked next to the branches.

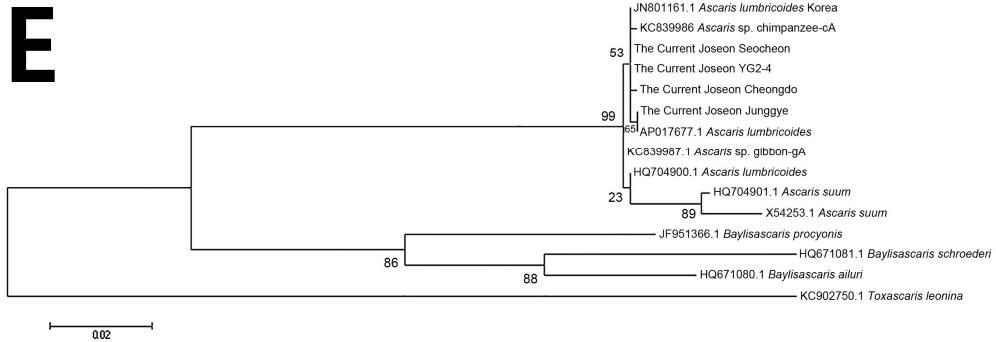

Figure 5-3. Maximum likelihood tree of *Ascaris* (E) concatenated sequences of mitochondrial DNA regions cyt b, CO1, and NAD1. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are marked next to the branches.

CHAPTER 2

Genetic analysis of small subunit ribosomal RNA, internal transcribe spacer 2 and ATP synthase subunit 8 of *Trichuris trichiura* ancient DNA retrieved from the 15th to 18th century Joseon Dynasty mummies' coprolites

Introduction

The infection of *Trichuris trichiura* is a very common intestinal helminthic disease, infecting about 800 million people worldwide (de Silva et al., 2003, Manz et al., 2017). Many researchers have thus attempted to accurately diagnose *T. trichiura* through DNA analysis (Cutillas et al., 2009; Areekul et al., 2010; Oh et al., 2010; Liu et al., 2012; Hawash et al., 2015; Meekums et al., 2015). The molecular diagnoses of *T. trichiura* have been conducted on small subunit ribosomal RNA (SSU rRNA) (Areekul et al., 2010) or internal transcribe spacer 2 (ITS2) (Cutillas et al., 2007, 2009; Meekum et al., 2015; Xie et al., 2018). Liu et al. (2012) and Hawash et al. (2015) also characterized the complete mitochondrial (mt) genome of *T. trichiura* and *T. suis*, exhibiting high variability in the ATP synthase subunit 8 (ATP8) of *Trichuris* spp. mtDNA.

Similar studies have been performed in the field of paleoparasitology as well. The earliest aDNA analysis was the determination of *T. trichiura* SSU rRNA sequence retrieved from the mid-18^{th} century human remains of South Korea (Oh et al., 2010). Since then, more studies have been conducted on the ancient specimens from different countries. Briefly, Myšková et al. (2014) amplified *Trichuris* SSU rRNA gene using the 18^{th}-19^{th} century soil

sediments collected from the excavation site in Czech Republic. Søe et al. (2015, 2018) also reported *T. trichiura* mtDNA sequences using the 5th to 18th century soil sediments from Denmark, Bahrain, the Netherlands and Lithuania.

Despite these efforts, the obtained data are still not enough to conclude the genetic characteristics of *T. trichiura* in a wide spatiotemporal perspective. Actually, *T. trichiura* aDNA samples analyzed so far were very few; and the paleoparasitological specimens were mainly collected from very limited regions (Oh et al., 2010; Myšková et al., 2014; Søe et al., 2015, 2018). Consequently, I was very hard to conjecture the phylogenetic relationship of *T. trichiura* in a geo-historical scope. Fortunately, over the past several years, I was able to collect the Korean mummies' feces specimens in which the presence of *Trichuris* eggs was microscopically identified (Seo et al., 2014, 2017). Utilizing these ancient coprolites, in this study, I tried to analyze SSU rRNA, ITS2 and ATP8 of *T. trichiura* aDNA, characterizing the paleogenetics of *T. trichiura* that was prevalent in pre-20th century Korean society.

Materials and Methods

The samples examined in this study were collected from the 15th to 18th century mummies of Joseon Dynasty (n=9; Jinju, Mungyeong, Junggye, Waegwan, YG2-4, YG2-6, SN2-19-1, SN2-19-2, and Seocheon). They were ancient feces obtained from mummy intestine (Jinju, Myngyenong, Junggye, and Seocheon); or the sediments precipitated upon the hipbones of other half-mummified cases (Waegwan, YG2-4, YG2-6, SN2-19-1, and SN2-19-2). Before aDNA analyses, microscopic examination confirmed the presence of *Trichuris* eggs in each ancient specimen. I followed *the Criteria of Authentication* that was previously recommended for authentic aDNA analysis (Hofreiter et al., 2001; Willerslev and Cooper, 2005). I wore masks, protection gloves, head caps, and gowns for each lab procedure. Experiment was done in our aDNA lab facility that has been maintained in accordance with *the Criteria of Authentication*.

Samples were treated with 1 ml lysis buffer (EDTA 50 mM, pH 8.0; SDS 1%; 0.1 M DTT; 1mg/ml of proteinase K) at 56℃. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1); and then was treated again with chloroform/isoamyl alcohol (24:1). DNA was isolated and purified using a QIAmp PCR

purification kit (Qiagen, Hilden, Germany). The purified DNA was eluted in 40 μl of EB elution buffer (Qiagen, Hilden, Germany). The PCR primers for *T. trichuira* SSU rRNA, ITS2, and ATP8 were generated by Integrated DNA Technologies, Inc. (Iowa City, IA, USA). Table 3 summarizes the information of my PCR primer sets.

DNA was quantified by NanoDrop™ ND-1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA). Extracted DNA (10 μl) was treated with 1 unit of uracil-DNA-glycosylase (New England Biolabs, MA, USA) at 37℃, and a part of it (40 ng) was mixed with the reagent premix containing 1X AmpliTaq Gold® 360 Master Mix (Life Technologies, CA, USA) and 10 pmol of each primer. The experiment conditions of PCR were as follows: pre-denaturation at 94℃ for 10 min; 45 cycles of denaturation at 94℃ for 45 sec, annealing at 55 or 58℃ for 45 sec, extension at 72℃ for 45 sec, and final extension at 72℃ for 10 min. The PCR products were separated on 2.5% agarose gel (Invitrogen, CA, USA). Negative controls (extraction controls) were also applied to the same electrophoresis. After the agarose gel was stained with ethidium bromide, it was photographed by a Vilber Lourmat ETX-20.M equipped with Biocapt software (Vilber Lourmat, Collégien, France).

Cloning and sequencing was performed for amplified PCR

product of the expected size. After DNA contained in the amplified band was extracted by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), bacterial transformation of amplified DNA product was performed using pGEM-T Easy Vector system (Promega Corporation, Madison, USA). Transformed bacteria were grown in the agar plates containing ampicillin (100μg/ml), 0.5 mM IPTG and X-GAL (40 μg/μl). When selected colonies were grown in LB media again for 12 hrs, plasmid was harvested with QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Sequencing was done on each strand using an ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Waltham, USA), in the 3730xl Automated Sequencer (Applied Biosystems, Waltham, USA).

To get the consensus sequence, I repeated cloning and sequencing for specific PCR amplicons. The multiple sequence alignment was performed by Clustal W implemented in MEGA7 program (Kumar et al., 2016). I obtained consensus sequence of each DNA region based on the aligned clone sequences. To retrieve the sequences in National Center for Biotechnology Information (NCBI) homologous to my consensus sequence, I used NCBI BLAST tool (Altschul et al., 1997).

As for *T. trichuira* SSU rRNA, ITS2, and ATP8, I inferred

the phylogenetic relationship among taxa by the Maximum likelihood (ML) method (Saitou and Nei, 1987) implemented in MEGA7 program (Kumar et al., 2016). Parameters selected for ML method were Kimura 2-parameter model (SSU rRNA and ATP8; Kimura, 1980) or Tamura 3-parameter (ITS2; Tamura, 1992) for Model/Method; Uniform Rates for Rates among Sites; selecting complete deletion for gaps/missing data treatment; and Nearest-Neighbor-Interchange (NNI) for ML Heuristic Method. To estimate the reliability of the tree, I did bootstrap test of MEGA7 (Hall, 2013). Number of bootstrap replicates was 1000. ML tree for *T. trichuira* SSU rRNA, ITS2 and ATP8 region sequences with bootstrap values were then made.

Table 3. List of primers used for amplification of *T. trichiura* DNA in this study.

Locus	Primer set	Sequence (5' → 3')	Annealing Temp. (°C)	PCR cycles	Amplicon size (bp)
SSU rRNA	T.tri-18s F1	TCC CAG ATC GGG GAG GTA GTG	55	45	194
	T.tri-18s R1	CGC AAT CCA ACT ACG AGC GGT			
	T.tri-18s F2	AAT CTA TTG GAG GGC AAG TCT GG	58		188
	T.tri-18s R2	TCA CTG AAG AGC ATC CAG GG			
	T.tri-18s F3	CCT AAG CAG GAG TCG TTC CG	58		176
	T.tri-18s R3	TAG CAC TGA GGC CAT CTT TC			
ITS2	T.tri-ITS2 F1	AAG ATG TCG ACG CTA CGC CTG	58		156
	T.tri-ITS2 R1	CTC CGC CTA CCA GTT GCC ATC			
	T.tri-ITS2 F2	GCA CCG GAC AAA CCT GCA TCC	58		170
	T.tri-ITS2 R2	GCT CAA CTG CTG TAC GCC GAC			
	T.tri-ITS2 F4	AGT CAG CGT AGG GCG AAG ACT	58		175
	T.tri-ITS2 R4	AGC AAG GCG AAC GGT TTC GTC G			
	T.tri-ITS2 F7	CGT AGC AGC AAG TGT TCG TCG	58		198
	T.tri-ITS2 R7	ACC GCT AGT TGA CGA ACG GCA			
ATP8	T.tri-ATP8 F1	AAC TCA AAC ATT GGA TTG TCA	55		135
	T.tri-ATP8 R1	TGG GAA TTT TAA GTC CAA CA			
	T.tri-ATP8 F2	AGC TAT CTT TTT ATT GTT GAA TGG	55		162
	T.tri-ATP8 R2	AGA ATT CTT AGA AGA GCG AAA AA			

Results and Discussion

In my electrophoresis results, the amplified products of the *Trichuris* SSU rRNA, ITS2 and ATP8 regions were detected in Seocheon and YG2-4 samples, whereas the negative controls (extraction controls) did not show any bands of PCR amplification (Figure 6). SSU rRNA and ITS2 specific bands were detected in Seocheon and YG2-4 samples while the ATP8 region showed specific bands only in Seocheon sample (Figure 6). By the cloning and sequencing trials, 9-12 clones were successfully sequenced for each amplicon (Figure 7).

The *T. trichiura* SSU rRNA sequences of two ancient samples (Seocheon and YG2-4) were identical to each other, being also the same as the *T. trichuira* SSU rRNA sequence (Access No. GU339222.1) reported from another Korean mummy (SN19-2) (Oh et al., 2010). The ancient consensus sequences (of Seocheon and YG2-4) exhibited very high similarities to GenBank *T. trichiura* SSU rRNA sequences which were reported from Japan (Coverage 100%, Ident 100%, AB699090.1), Thailand (Coverage 100%, Ident 100%, GQ352555.1; Coverage 100%, Ident 99.71%, GQ352553.1), and ancient Denmark (Coverage 81%, Ident 100%, KM365014.1). I also found parasite species showing high similarities to my *T.*

trichiura SSU rRNA sequences, including *T. suis* (Coverage 100%, Ident 97.97%, HF586905.1), *T. leporis* (Coverage 100%, Ident 95.64%, KR025525.1), *T. skrjabini* (Coverage 100%, Ident 95.64%, HF586912.1), *T. ovis* (Coverage 100%, Ident 95.35%, HF586911.1) and *Stilesia* sp. (Coverage 100%, Ident 95.07%, KR082010.1) (Figure 8A).

In case of the *T. trichuira* ITS2, the sequences of two ancient mummy samples (Seocheon and YG2-4) were also identical to each other. In BLAST searching, though the GenBank did not include the taxa 100% identical to my Seocheon and YG2-4 ITS2 sequences, highly similar *T. trichiura* ITS2 sequences were reported from Turkey (Coverage 100%, Ident 99.77%, KC877992.1), China (Coverage 100%, Ident 99.77%, AM992984.1; Coverage 100%, Ident 98.39%, AM992993.1; Coverage 100%, Ident 97.24%, AM992998.1) and Uganda (Coverage 97%, Ident 99.77%, JN181851.1; Coverage 97%, Ident 99.30%, JN181849.1; Coverage 97%, Ident 98.12%, JN181845.1; Coverage 97%, Ident 96.01%, JN181858.1; Coverage 97%, Ident 94.48%, JN181855.1). I also found non-*T. trichiura* species also showing similarities to Seocheon and YG2-4 ITS2 sequences: *T. suis* (Coverage 75%, Ident 65.61%, MG656443.1; Query cover 71%, Ident 64.67%, JN181808.1) and *T. colobae* (Coverage 81%, Ident 66.77%,

FM991956.1) (Figure 8B).

Next, ATP8 sequence (Seocheon) also exhibited very high similarity to the GenBank *T. trichiura* ATP8 sequences reported from China (Coverage 100%, Ident 98.98%, GU385218.1), Japan (Coverage 100%, Ident 92.93%, AP017704.1) and Uganda (Coverage 100%, Ident 73.13%, KT449826.1). The degree of similarity was relatively lower in case of *T. trichiura* reported from Europe: Kempen of ancient Netherlands (Coverage 100%, Ident 72.14%, KY368766.1; Coverage 100%, Ident 71.64%, KY368767.1), Viborg of ancient Denmark (Coverage 100%, Ident 71.64%, KY368774.1) and Zwolle of ancient Netherlands (Coverage 100%, Ident 71.64%, KY368765.1) (Figure 8C).

The phylogenetic trees of *T. trichuira* SSU rRNA, ITS2 and ATP8 are summarized in Figure 3. Phylogenetic analysis of *T. trichiura* re-confirmed the results of BLAST searching. In brief, every *T. trichiura* sequence of SSU rRNA, including the sequences of my Joseon specimens, was clearly distinct from the other species of genus *Trichuris* (*T. suis, T. colobae, T. leporis,* and *T. muris*) (Figure 9A). Similarly, as for the ITS2, phylogenetic tree also showed that *T. trichiura* and other genus *Trichuris* species (e.g. *T. suis*, *T. colobae*, and *T. muris*, etc.) were clustered into different clades (Figure 9B). Finally, in case of ATP8 region, Seocheon

sequence was clustered together with GenBank *T. trichiura* ATP8 sequences of China (GU385218.1) and Japan (AP017704.1) while it was distinct from *T. trichiura* cluster of Uganda (KT449826.1) and ancient Europe (KY368765–KY368774) (Figure 9C).

Taken together, in this study, I successfully characterized the SSU rRNA, ITS2 and ATP8 sequences of *T. trichiura* aDNA from the 16th to 18th century Korean mummies. I found that each *T. trichiura* sequence of Korean mummies belonged to a separate cluster that was evidently distinct from the other species of genus *Trichuris* reported so far in GenBank. I thus proved the usefulness of aDNA analysis in differential diagnosis of *T. trichiura* even in case that the parasite eggs found in archaeological sites were morphologically indeterminate for a microscopic identification of the species. Considering the rarity of genetic information about *T. trichiura* reported so far, I need more *T. trichiura* aDNA sequences with a wide geo–historical scope to make our knowledge about the genetic history of *T. trichiura* much improved.

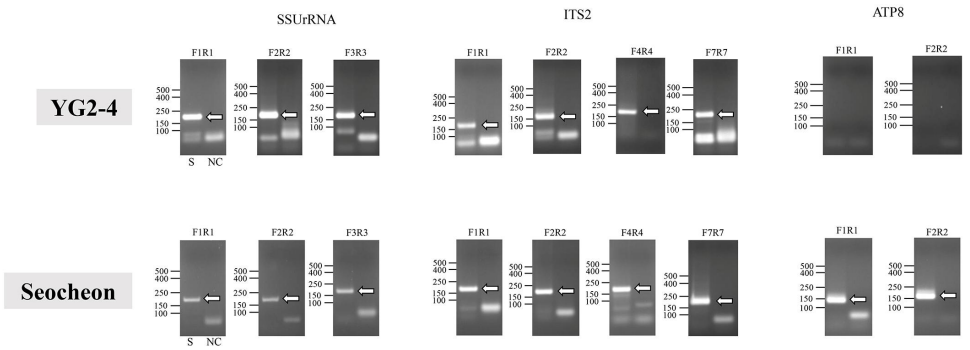

Figure 6. Agarose gel electrophoresis for the PCR amplicons of *T. trichiura* aDNA from Seocheon and YG2-4 mummies. Note specific amplicons 1 (194bp), 2 (188bp), 3 (176bp), of SSU rRNA region; 1 (156bp), 2 (170bp), 3(175bp), 4 (198bp) of ITS2 region; and 1 (135bp), 2 (162bp) of ATP8 region.

Seocheon

YG2-4

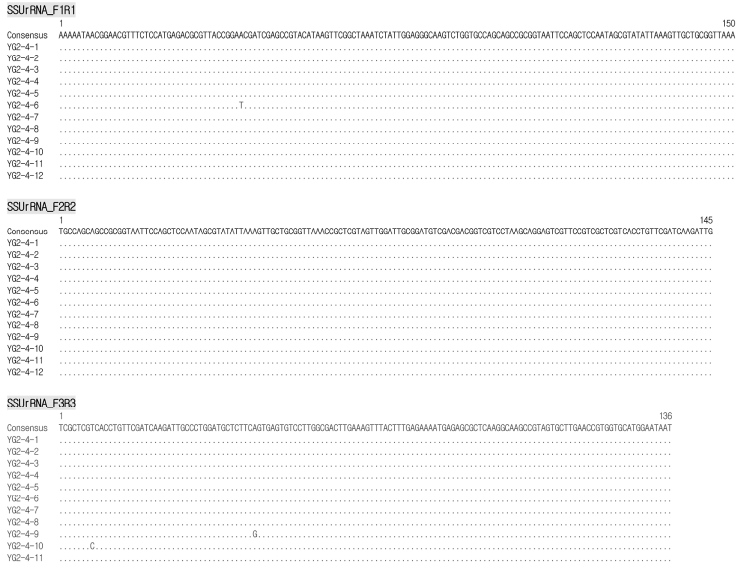

Figure 7-1. Aligned clone sequences of SSU rRNA fragments from Joseon Dynasty mummies.

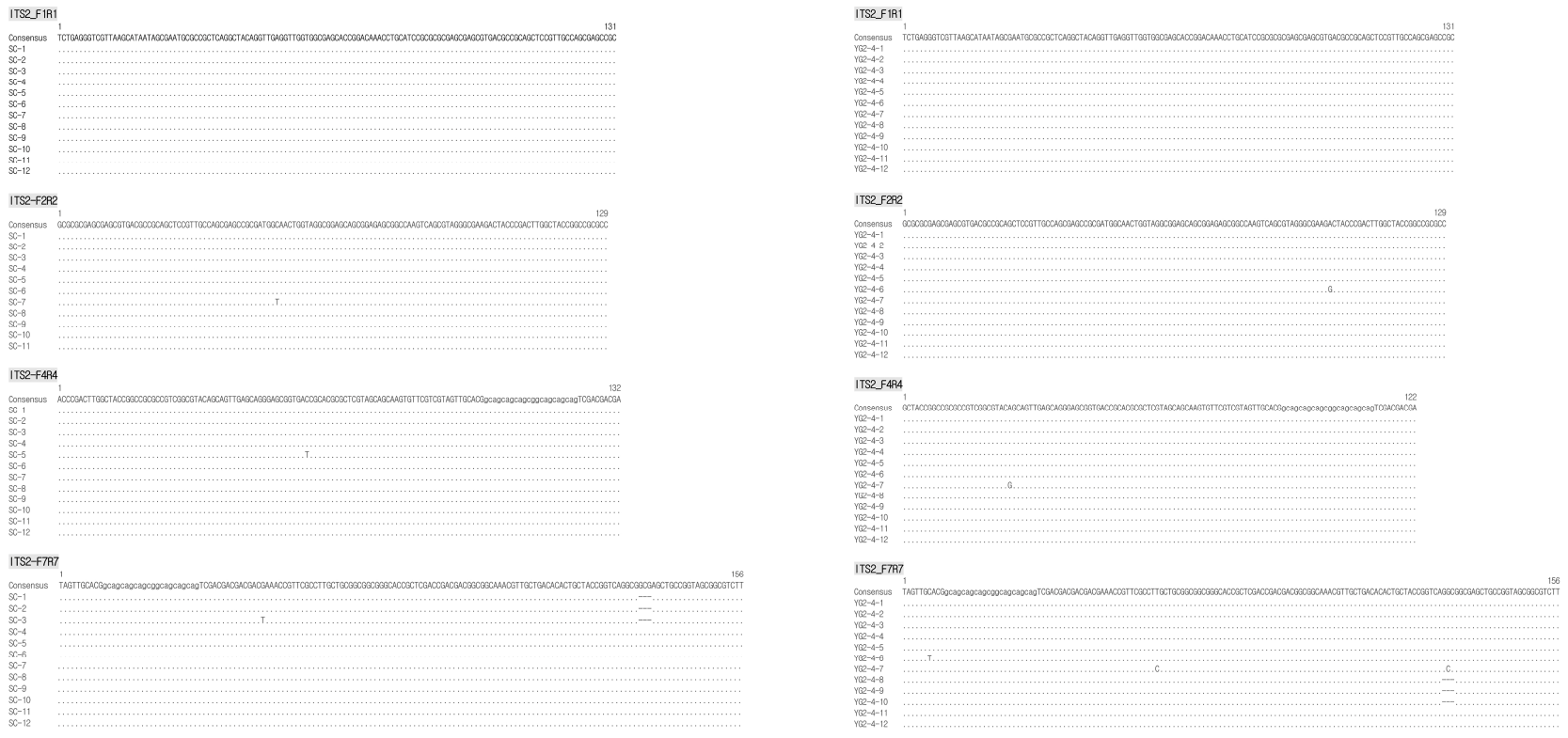

Figure 7-2. Aligned clone sequences of ITS2 fragments from Joseon Dynasty mummies.

Seocheon

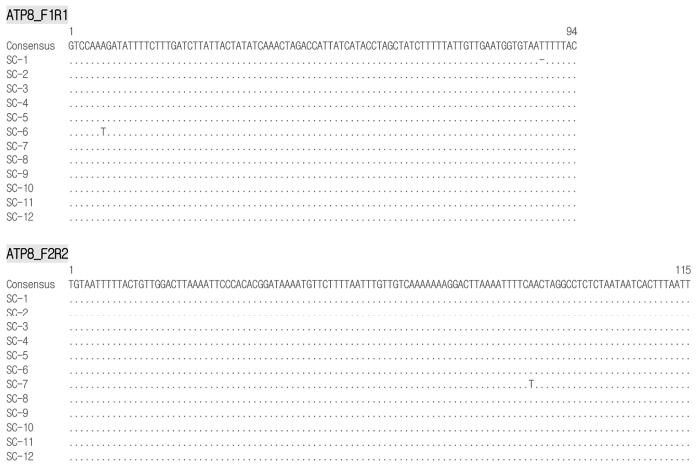

Figure 7-3. Aligned clone sequences of ATP8 fragments from Joseon Dynasty mummy.

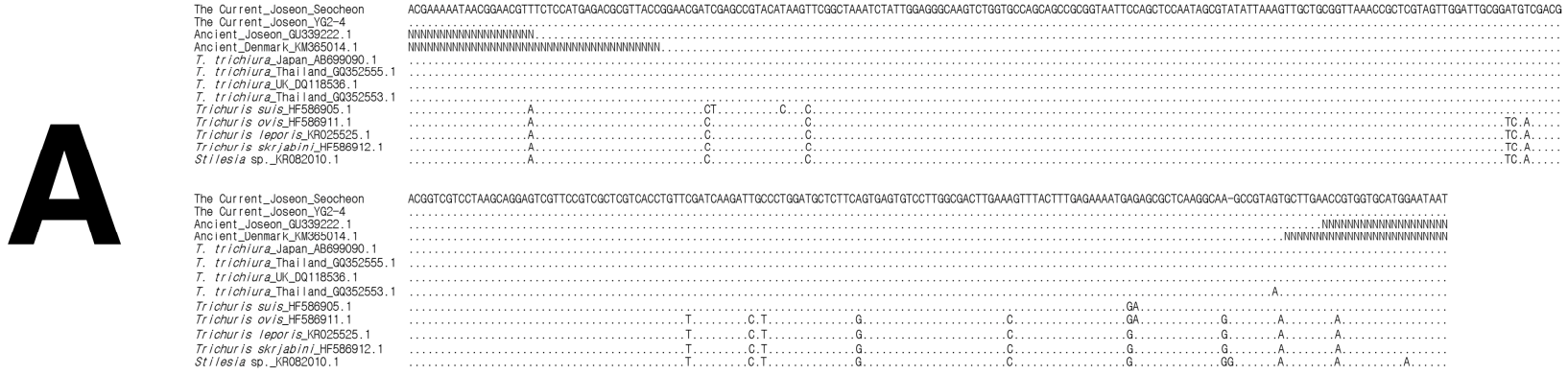

Figure 8-1. BLAST comparison of consensus *T. trichiura* sequences from Seocheon and YG2-4 mummies with the other sequences retrieved at GenBank. (A), (B) and (C) are SSU rRNA, ITS2, and ATP8 region sequences, respectively.

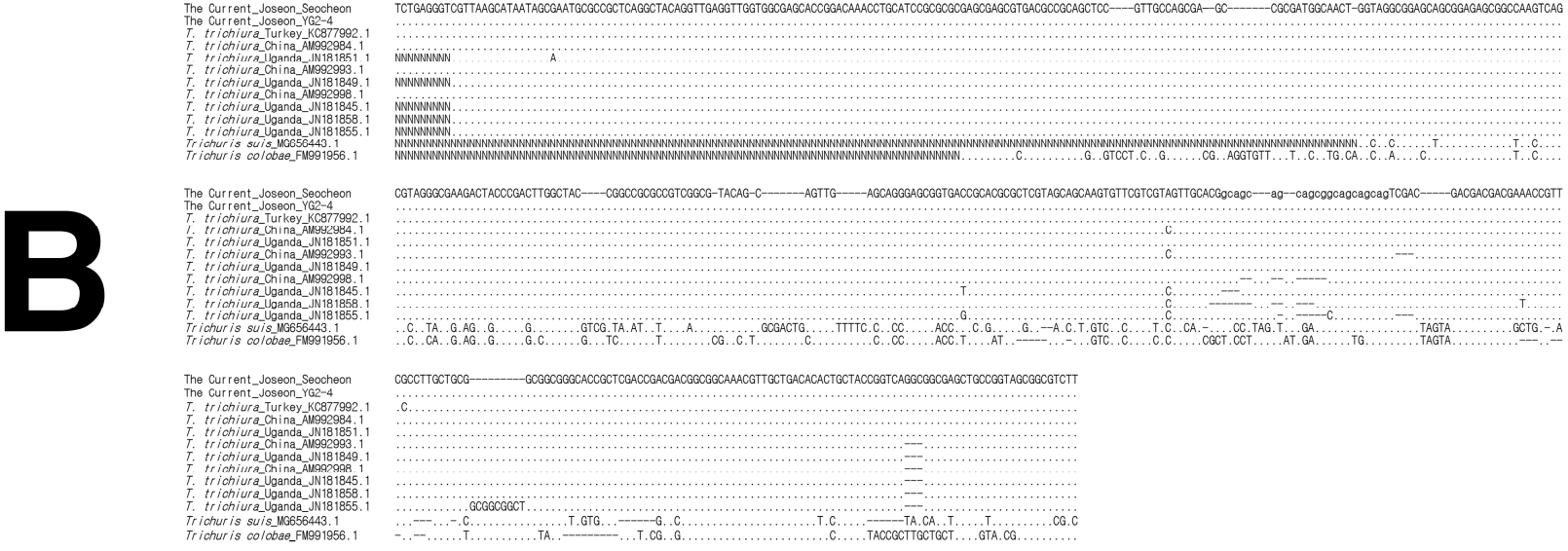

Figure 8-2. BLAST comparison of consensus *T. trichiura* sequences from Seocheon and YG2-4 mummies with the other sequences retrieved at GenBank. (A), (B) and (C) are SSU rRNA, ITS2, and ATP8 region sequences, respectively.

C

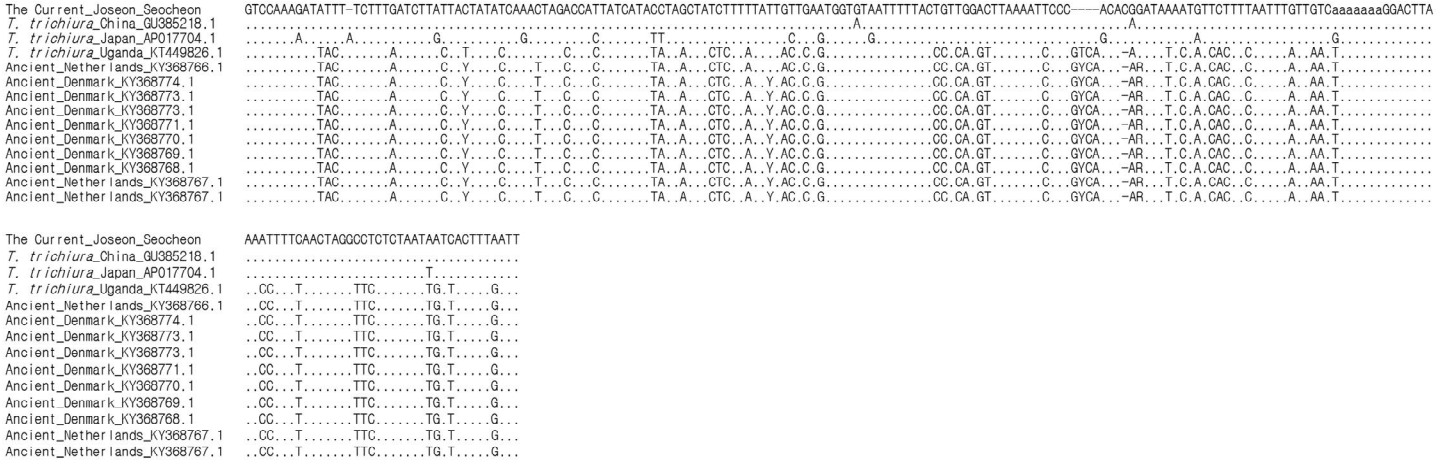

Figure 8-3. BLAST comparison of consensus *T. trichiura* sequences from Seocheon and YG2-4 mummies with the other sequences retrieved at GenBank. (A), (B) and (C) are SSU rRNA, ITS2, and ATP8 region sequences, respectively.

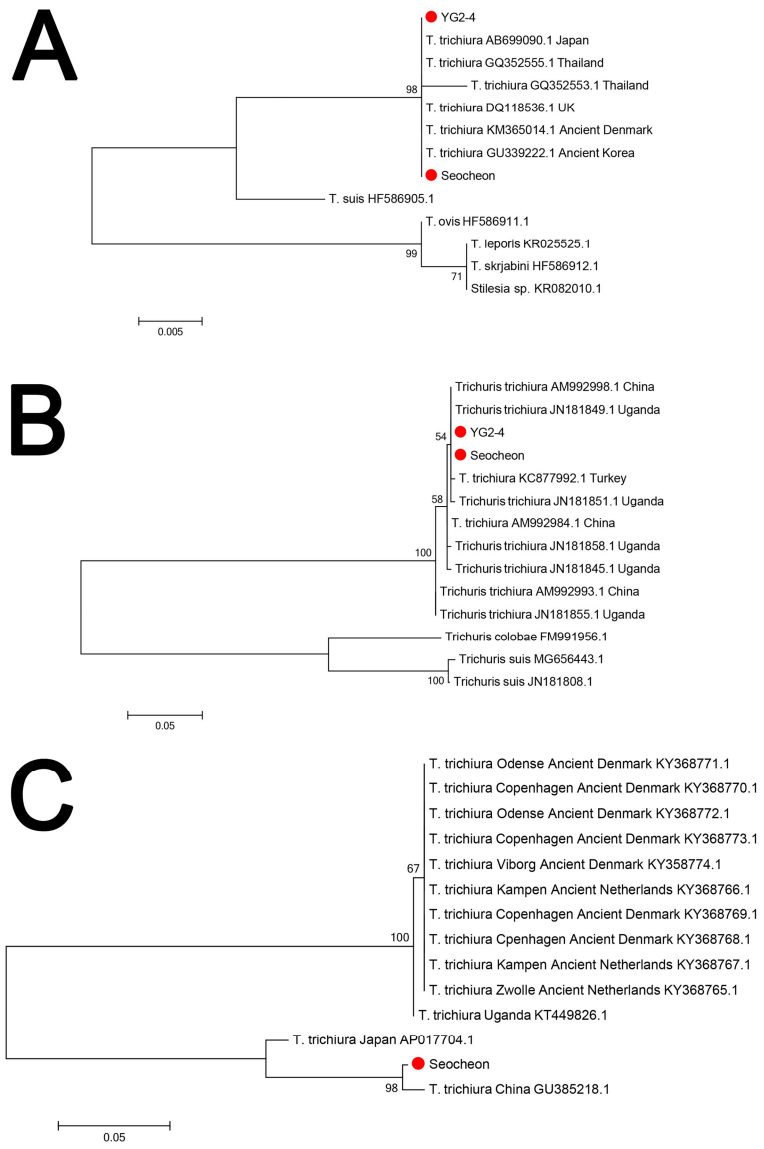

Figure 9. Maximum likelihood tree of *Trichuris* (A) SSU rRNA, (B) ITS2, and (C) ATP8 DNA region. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are marked next to the branches. The *T. trichiura* aDNA sequences revealed in this study are represented by red dots.

CHAPTER 3

DNA analysis of CO1 and ITS2 regions using *Paragonimus westermani* eggs remained in the ancient coprolites from Joseon Dynasty mummies

Introduction

Paragonimiasis occurs in definitive hosts by the ingestion of infected intermediate hosts (Blair et al., 2016). In general, *Paragonimus* eggs hatch before entering snails (the first intermediate hosts). When the cercariae penetrate the crustaceans such as crayfishes or crabs (the second intermediate hosts), they encyst in gills, muscles, and viscera, developing into metacercariae. After definitive hosts (e.g. human) eat the freshwater crustaceans in raw, the metacercariae excyst in the small intestine, penetrating the intestinal wall. They then traverse the diaphragm, entering the peribronchiolar tissues of the lung where they mature into adults within 8–12 weeks (Bogitsh et al., 2005).

The genus *Paragonimus* constitutes a species-rich group from the tropical regions in South and Southeast Asia, Africa and America as well as the temperate zone of North America and East Asia (Blair et al., 1999). Almost nominal 50 species and subspecies of *Paragonimus* have been reported so far and approximately 16 species have been revealed to cause human diseases (Blair et al., 2016). Actually, paragonimiasis mainly affect the lung. The adult worms stimulate the formation of worm capsule. When the capsule

ulcerates, the clinical symptoms of cough, blood-tinged sputum, pulmonary pain etc. could be developed. Paragonimiasis could also be induced by the migration of young adult worms to the ectopic organs (Choi, 1990; Kim et al., 2004; Bogitsh et al., 2005; Koh et al., 2012; Shin et al., 2012). Among genus *Paragonimus*, the most common species is *P. westermani*, the oriental lung fluke mainly reported from Korea, China, Taiwan, Japan and the Philippines (Kerbert, 1878; Devi et al., 2013; Blair et al., 2016). Scholars estimated that over 20 million people are currently infected with *P. westermani* worldwide (Zarrin-Khameh et al., 2008; Blair et al., 2016).

Recently, researchers have attempted to reveal the genetic characteristics of *P. westermani* through DNA analysis (Iwagami et al., 2003, 2008; Binchai et al., 2007; Sugiyama et al., 2007; Doanh et al., 2009; Prasad et al., 2009; Devi et al., 2010, 2013; Blair et al., 2016). By phylogenetic analyses of cytochrome c oxidase subunit I (CO1) and internal transcribed spacer 2 (ITS2) DNA regions, *P. westermani* were clustered into at least two groups (East Asia and South/Southeast Asia), in association with geographically distinct distributions (Iwagami et al., 2003; Binchai et al., 2007; Doanh et al., 2009; Devi et al., 2013; Blair et al., 2016). However, as for *Paragonimus* spp. found in archaeological sites, though eggs have

been detected in the archaeological samples, very few parasitological reports about paleogenetics have been made so far. Heretofore, the one and only report was our previous study on ITS2 DNA sequence of *P. westermani* eggs obtained from a 17th century Korean mummy (Shin et al., 2012). In that, the aDNA sequence of *P. westermani* was very similar to that of modern *P. westermani* reported in East Asia, but were genetically distinct from the *P. westermani* sequences of Southeast Asia (Shin et al., 2012).

Nevertheless, our previous study was performed for only one sample and with a single genetic marker (ITS2). Actually, over the past several years, the genetic information of another *P. westermani* genetic marker (CO1) has been continuously accumulated by multiple researchers (Iwagami et al., 2003, 2008; Binchai et al., 2007; Doanh et al., 2009; Devi et al., 2010). Our paleoparasitological studies have also microscopically detected *Paragonimus* eggs in many more coprolite specimens (n=6) from the 15th– to 18th–century Korean mummies (Seo et al., 2014, 2017). In this regard, I tried to examine newly collected *P. westermani* eggs with multiple genetic markers (CO1 and ITS2) in order to get more comprehensive information about the evolutionary history of *P. westermani*.

Materials and Methods

The samples used in this study were acquired from the 15th to 18th century Joseon mummies (Cheongdo, Dangjin, Hwasung, YG2-4, YG2-6, and Yongin). The specimens were ancient feces from the intestines of mummies (Cheongdo and Hwasung); or the organic materials compiled upon the hipbones of the half-mummified cases (Dangjin, YG2-4, YG2-6, and Yongin).

To make my aDNA work authentic, I followed the Criteria of Authentication by Hofreiter et al. (2001). During aDNA analysis, I wore head caps, masks, protection gloves, and gowns. All the tools used in this study were sterilized before use. I also used specialized facilities that were exclusively dedicated to aDNA analysis (Ho and Gilbert, 2010). I did experiments in our aDNA lab facility that is set up in accordance with the Criteria (Hofreiter et al., 2001). The Institutional Review Board (IRB) of Seoul National University Hospital confirmed that my aDNA analyses could be exempt from the board review (IRB No. 2017-001). I also followed the Vermillion Accord on Human Remains of World Archaeological Congress (Fforde, 2014).

For DNA extraction, I followed the method described in our previous report (Kim et al., 2011). In brief, the samples (0.3g)

were incubated at 56℃ in 1 ml of lysis buffer (pH 8.0; including 1% SDS; 0.1 M DTT; 50 mM of EDTA; 1 mg/ml of proteinase K) for 24 hrs. After DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), it was then treated with chloroform/isoamyl alcohol (24:1). DNA isolation and purification were performed using a QIAmp PCR purification kit (Qiagen, Hilden, Germany). The purified DNA was eluted in 40 μl of EB elution buffer (Qiagen, Hilden, Germany). Primers for *P. westermani* CO1 and ITS2 regions were generated in Integrated DNA Technologies, Inc. (Iowa City, IA, USA). The information of the used primers is summarized in Table 4.

DNA quantification was done by NanoDrop™ ND-1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA). Extracted DNA (10 μl) was treated at 37℃ with 1 unit of uracil-DNA-glycosylase (New England Biolabs, MA, USA) for 30 min. It (40 ng) was then mixed with the reagent premix containing 10 pmol of each primer and 1X AmpliTaq Gold® 360 Master Mix (Life Technologies, CA, USA). PCR conditions were as follows: pre-denaturation at 95℃ for 10 min; 45 cycles of denaturation at 95℃ for 30 sec, annealing at 54-60℃ for 30 sec, extension at 72℃ for 30 sec; and final extension at 72℃ for 10 min. The PCR products were separated on 2.5% agarose gel (Invitrogen, CA, USA), and

then stained with ethidium bromide. Negative controls (extraction controls) were also applied to the electrophoresis at the same time. Electrophoresis results were photographed using a Vilber Lourmat ETX-20.M equipped with Biocapt software (Vilber Lourmat, Collégien, France).

The PCR amplicons were isolated using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The transformation of bacteria was done with the pGEM-T Easy Vector system (Promega Corporation, Madison, USA). Transformed bacteria were grown in agar plate containing X-GAL (40 μg/μl), ampicillin (50 μg/ml), and 0.5 mM IPTG for the next 14 hrs. After selected colonies were grown in LB media for 12 hrs, the purification of cultured bacteria was performed by a QIAprep® Spin Miniprep kit (Qiagen, Hilden, Germany).

Sequencing on each amplified strand was done using an ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Waltham, USA) and the 3730xl Automatic Sequencer (Applied Biosystems, Waltham, USA). The obtained DNA sequences were aligned by MEGA7 program (Kumar et al., 2016). The consensus sequences were compared to those available in GenBank by NCBI/BLAST tools (Altschul et al., 1997). Web browser module and Alignment Explorer of MEGA7 were used for

retrieving sequences homologous to those of interest from NCBI GenBank database.

The evolutionary relationship of *P. westermani* and the other taxa from NCBI GenBank was inferred by the Phylogeny Reconstruction analysis implemented in MEGA7 (Kumar et al., 2016). I used Maximum Likelihood (ML) method. Selected parameters of Model/Method were Tamura-Nei model (Tamura, 1992) for CO1 or Kimura 2-parameter model (Kimura, 1980) for ITS2; selecting Complete deletion for Gaps/Missing data treatment; Uniform Rates for Rates among Sites; and Nearest-Neighbor-Interchange (NNI) for ML Heuristic Method. To estimate the reliability of the tree, I did bootstrap test (Hall, 2013). The number of bootstrap replicates was 1000.

Table 4. List of primers used for amplification of *P. westermani* DNA in this study

Region	set	Primer	5' to 3'	Annealing Temp. (◦C)	Length (bp)
CO1	CO1-1	COI-1F	GGG CAT CCG GAG GTG TAT GT	54	106
		COI-1R	TTC GGG TAC TAC GGG CTG G		
	CO1-2	COI-2F	CTG ACC AAC AAC GAT TCC T	54	150
		COI-2R	TCC CGT GAC AGA ACT AAA GA		
	CO1-3	COI-3F	GTC TGG GTA GTG TTG TGT GG	54	136
		COI-3R	AGC ATG AAC AAC CAA GAG AA		
	CO1-4	COI-4F	TTA GTT CTG TCA CGG GAG TG	57	114
		COI-4R	GAA TTC ACC ACA AAA CAG GA		
	CO1-5	COI-5F	TTC TCT TGG TTG TTC ATG CT	54	183
		COI-5R	GAC GTA ATG AAA ATG AGC C		
ITS2	ITS2-1	ITS2-1F	GCG CAG CCA ACT GTG TGA A	57	133
		ITS2-1R	GGC GTC GCG ATA GTT TAT		
	ITS2-2	ITS2-2F	TTA ATG CGA ACT GCA TAC TG	54	169
		ITS2-2R	AAG ACC AGA TTG GGG AGA T		
	ITS2-3	ITS2-3F	GGT CGG CTT ATA AAC TAT CG	54	129
		ITS2-3R	CCC GAG TAT GTT AGG GAA A		
	ITS2-4	ITS2-4F	AAT CTG GTC TTG TGC CTG T	60	165
		ITS2-4R	AAA CCA CAG ATG AAG ACA GG		
	ITS2-5	ITS2-5F	GTG GCT CAG TGA ATG ATT TAT	54	170
		ITS2-5R	CCG CTT AGT GAT ATG CTT A		

Results and Discussion

To select the specimens used for this experiment, I screened all the mummy coprolite samples using PCR with *P. westermani* primers (CO1-1 and ITS2-1 in Table 4). In agarose gel electrophoresis, the amplified products specific for *P. westermani* primer sets CO1-1 (106bp) and ITS2-1 (133bp) were detected only in two samples (Cheongdo and YG2-4) while negative controls (extraction controls) did not exhibit any amplified bands (Figure 10). I thus used Cheongdo and YG2-4 mummy specimens for subsequent experiments.

To obtain the consensus sequences of CO1 and ITS2 regions, I repeated cloning and sequencing for each specific amplicon. By these trials, 9-12 clone sequences were successfully acquired for CO1 (390bp) and ITS2 (417bp) regions. Next, multiple sequence alignment was performed using Clustal W implemented in MEGA7. I then obtained consensus sequence after alignment of the cloned sequences (Figure 11). The consensus sequences of *P. westermani* CO1 and ITS2 regions from the Cheongdo and YG2-4 mummies were identical to each other (Figure 12).

By NCBI/BLAST tools, I compared the consensus sequences with those available in GenBank (Figure 12). BLAST searching

results are summarized in Table 5. Briefly, the current *P. westermani* CO1 sequences from Joseon mummies exhibited high similarities to *P. westermani* CO1 region reported from Korea (AF333280.1; AF540958.1), Japan (U97205.1; U97208.1), China (AY140680.1; U97209.1), Vietnam (FJ434988.1), India (JN656169.1; KM280646.1), the Philippines (U97213.1), Thailand (AB354224.1) and Sri Lanka (AY240940.1). I also found moderate similarities to the GenBank sequences of *P. siamensis* (AB354231.1), *P. paishuihoensis* (AB679289.1) and *P. mexicanus* (KC562301.1) (Figure 12A; Table 5).

In the case of the ITS2 region, the current Joseon *P. westermani* sequences exhibiting similarities to *P. westermani* ITS2 sequences of Korea (AF333277.1), Japan (U96907.1), China (KC417492.1; AB713404.1), India (AB938198.1; JN656208.1; DQ836243.1), Taiwan (U96908.1), Vietnam (LC144902.1; FJ434982.1), Malaysia (U96909.1), Thailand (AB354217.1; AF159604.1), and Sri Lanka (AY240942.1). Similar sequences were also found in the sequences of *P. siamensis* (AB354222.1), *Euparagonimus cenocopiosus* (AF159601.1), *P. skrjabini* (AB703444.1). *P. westermani* ITS2 sequence of Korean mummy previously reported by Shin et al. (2012) (JF500452.1) also showed 100% identity (Coverage 87%) (Figure 12B; Table 5).

Next, I performed phylogenetic analysis of *P. westermani* COI and ITS2 regions (Figure 13). Every *P. westermani* taxon of COI and ITS2 region was clearly distinct from the other species of genus *Paragonimus*. *P. westermani* sequences were clustered into several groups. In case of CO1 region, Group I included *P. westermani* sequences of East Asia (Korea, Japan, and China) while Group II from South and Southeast Asia (India, Philippines and Thailand) formed another cluster. I note that some sequences reported from India and Sri Lanka formed a separate cluster of the third separate group (Group III) (Figure 13A). *P. westermani* CO1 sequences of Korean mummies in this study evidently belong to the East Asia group. My result was similar to the phylogenetic analyses of Iwagami et al. (2003; 2008) and Devi et al. (2010; 2013) that *P. westermani* CO1 region was clustered into the groups of East, Southeast, and South Asia (Figure 13A).

Like CO1 region, *P. westermani* ITS2 sequences reported from East Asia (Korea, Japan and China; Group I), Southeast Asia (Thailand; Group II), South Asia (India; Group III) were also separately clustered in phylogenetic analysis (Figure 13B). The current *P. westermani* ITS2 sequences of Cheongdo and YG2-4 Korean mummies belonged to the East Asia group as well. Taken together, current phylogenetic tree of ITS2 region was generally

similar to the previous reports of Iwagami et al. (2003, 2008) and Devi et al. (2010, 2013) However, different from CO1 region, there was also a unique finding in this study of *P. westermani* ITS2 region. In brief, some of *P. westermani* ITS2 sequences of South Asia (India: AB938198.1, JN656208.1, JN656205.1) and Southeast Asia (Vietnam: LC144902.1, FJ434982.1) were clustered together with the East Asia group in this study (Figure 13B). Similar finding was rarely reported except for the research of Doanh et al. (2009).

According to the paleoparasitological estimation, *P. westermani* infection prevalence in Joseon society might have reached as high as 33.3% (Seo et al., 2014, 2017). Since Joseon people commonly enjoyed raw crayfish or raw crab dishes (Yun, 1960; Sohn et al., 2009; Shin et al., 2012), it is no wonder that they were heavily infected by *P. westermani*. In the present study, using the coprolite samples of Korean mummies, I successfully analyzed aDNA sequences of *P. westermani* CO1 and ITS2 regions. Considering very few cases reporting on *P. westermani* aDNA so far (Shin et al., 2012), the current study significantly expanded the existing gene pool of *P. westermani* paleogenetics. Nevertheless, I admit that aDNA reports of much wider geo-historical scope are still required to improve our knowledge about the exact evolutionary history of *P. westermani*.

Table 5. BLAST searching results for the coverage and percent identity of each taxon comparing to the consensus sequence of *P. westermani* CO1 and ITS2 from Korean mummies. GenBank accession numbers and geographical information are also indicated.

DNA region	Species	Coverage	Percent Identity	Accession Number	Geographical region
CO1	*P. westermani*	100%	100%	U97205.1	Japan
		100%	99%	AF333280.1	South Korea
		100%	99%	AF540958.1	South Korea
		100%	99%	U97208.1	Japan
		98%	99%	AY140680.1	China
		100%	98%	U97209.1	China
		99%	96%	FJ434988.1	Vietnam
		100%	93%	JN656169.1	India
		99%	86%	KM280646.1	India
		99%	90%	U97213.1	The Philippines
		99%	89%	AB354224.1	Thailand
		92%	84%	AY240940.1	Sri Lanka
	P. siamensis	99%	95%	AB354231.1	Thailand
	P. paishuihoensis	99%	84%	AB679289.1	Laos
ITS2	*P. westermani*	100%	100%	KC417492.1	China
		100%	100%	AB713404.1	China
		100%	100%	AB938198.1	India
		100%	99%	AF333277.1	South Korea
		100%	99%	JN656205.1	India
		100%	99%	JN656208.1	India
		100%	99%	U96908.1	Taiwan
		100%	99%	LC144902.1	Vietnam
		100%	99%	AB354217.1	Thailand
		100%	98%	DQ836243.1	India
		100%	98%	JN656199.1	India
		100%	97%	DQ351845.1	India
		100%	97%	AB354214.1	Thailand
		97%	99%	FJ434982.1	Vietnam
		87%	100%	JF500452.1	Ancient Korea
		87%	100%	U96907.1	Japan
		87%	98%	U96909.1	Malaysia
		87%	97%	AF159604.1	Thailand
		68%	95%	AY240942.1	Sri Lanka
	P. siamensis	100%	96%	AB354222.1	Thailand
	E. cenocopiosus	87%	93%	AF159601.1	China

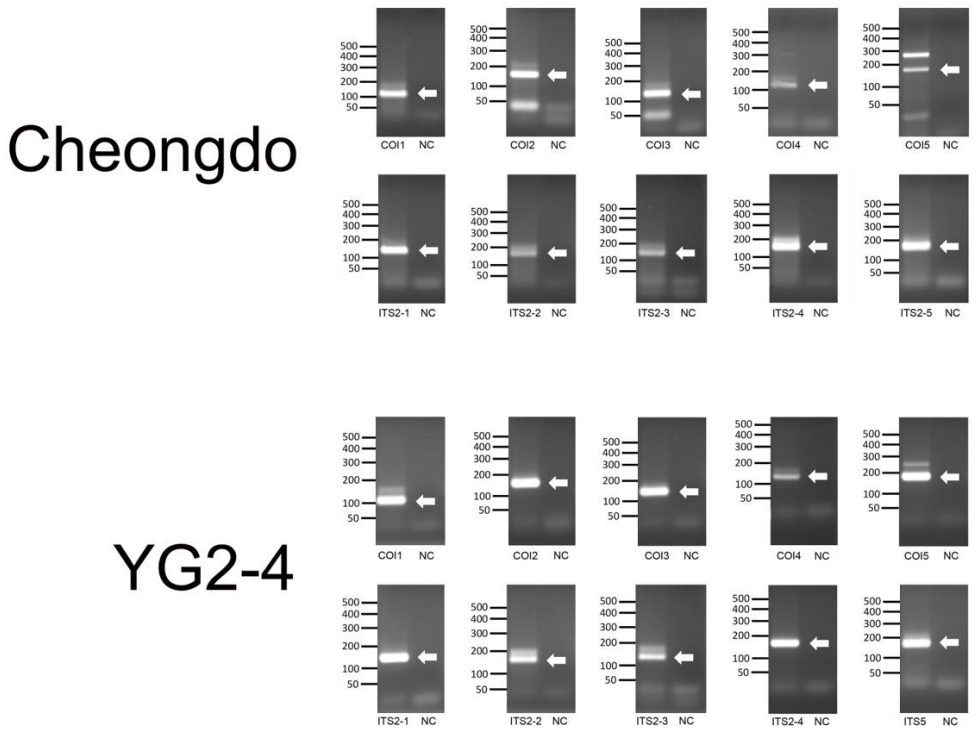

Figure 10. Agarose gel electrophoresis for the PCR amplicons of *P. westermani* aDNA from Cheongdo and YG2-4 mummies. Note specific amplicons 1 (106bp), 2 (150bp), 3 (136bp), 4 (114bp), 5 (183bp) of CO1 region; and 1 (133bp), 2 (169bp), 3(129bp), 4 (165bp), 5 (170bp) of ITS2 region.

Cheongdo

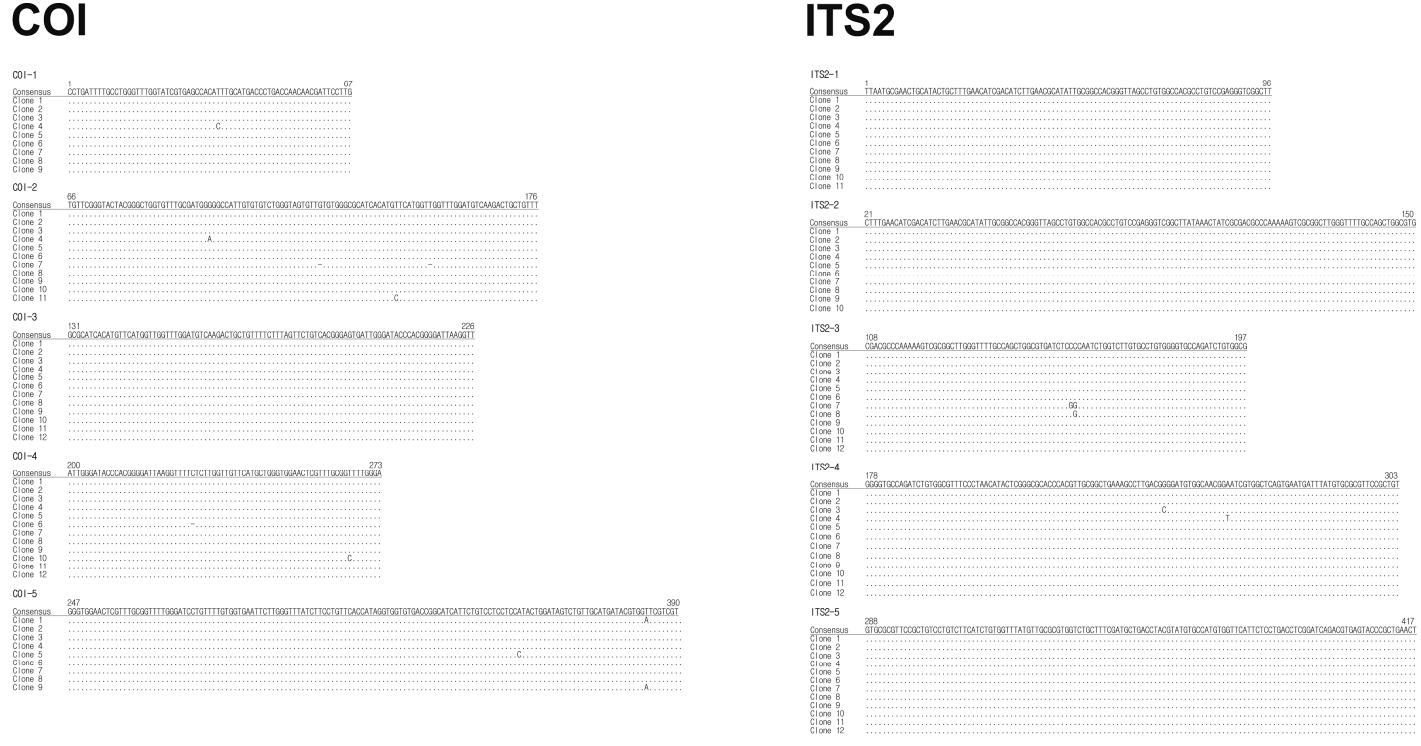

Figure 11-1. Sequence alignment of cloned PCR amplicons for the CO1 and ITS2 region of *P. westermani* aDNA from Cheongdo mummy.

Figure 11-2. Sequence alignment of cloned PCR amplicons for the CO1 and ITS2 region of *P. westermani* aDNA from YG2-4 mummy.

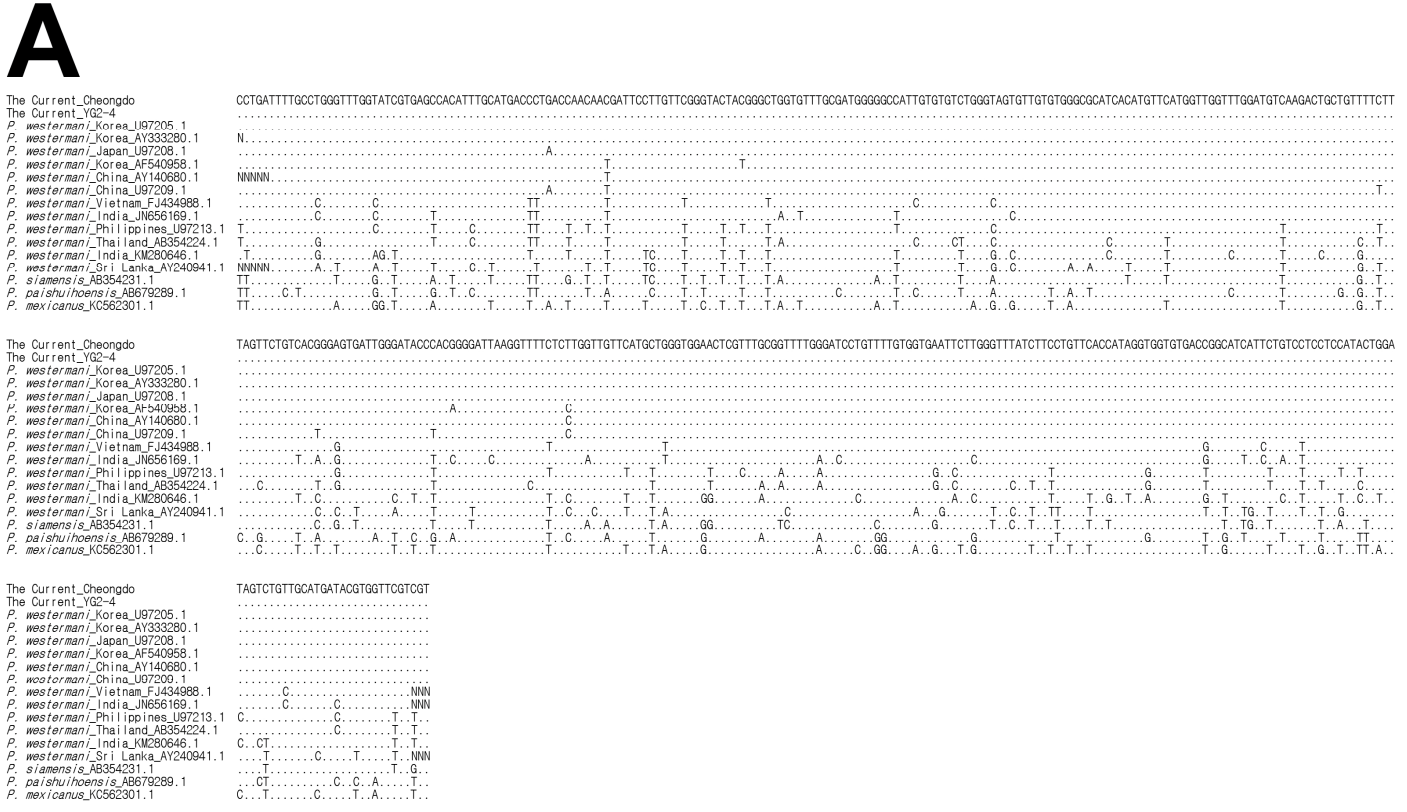

Figure 12-1. BLAST comparison of *P. westermani* consensus sequences from Cheongdo and YG2-4 mummies with the other sequences retrieved at GenBank. (A) and (B) are CO1 and ITS2 region sequences, respectively.

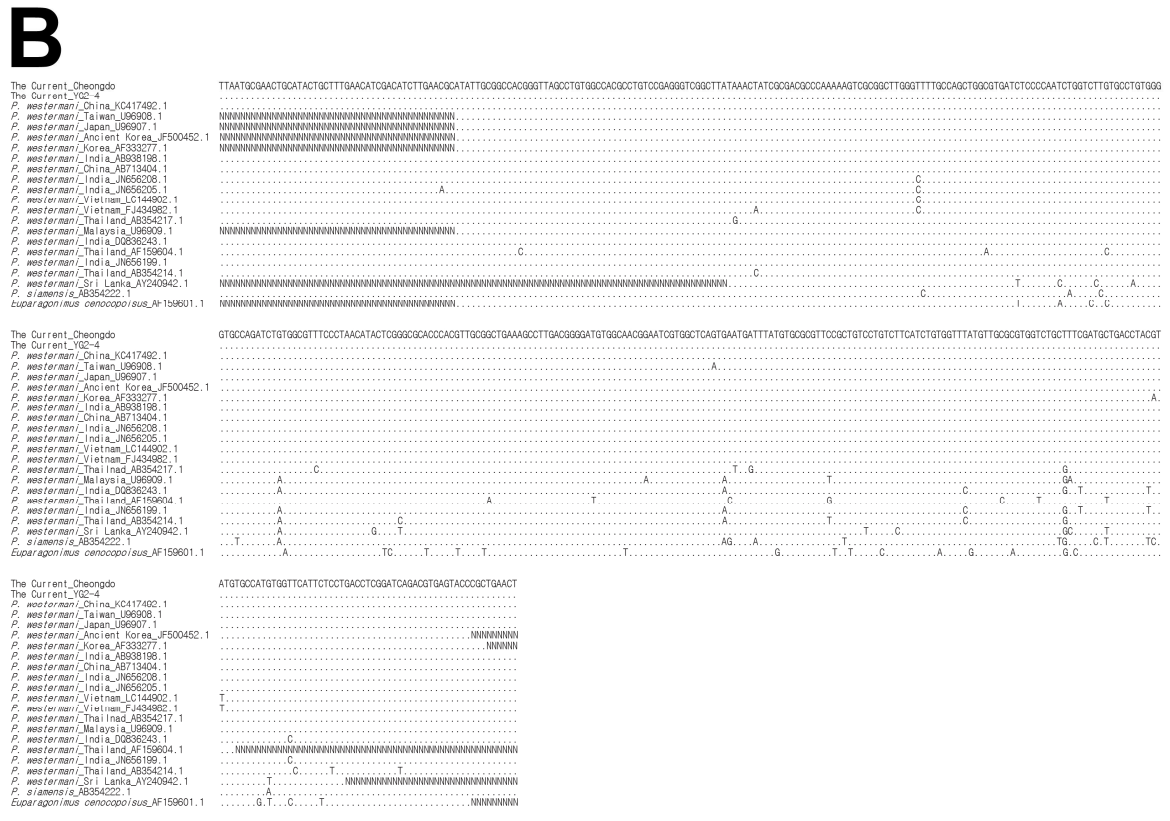

Figure 12-2. BLAST comparison of *P. westermani* consensus sequences from Cheongdo and YG2-4 mummies with the other sequences retrieved at GenBank. (A) and (B) are CO1 and ITS2 region sequences, respectively.

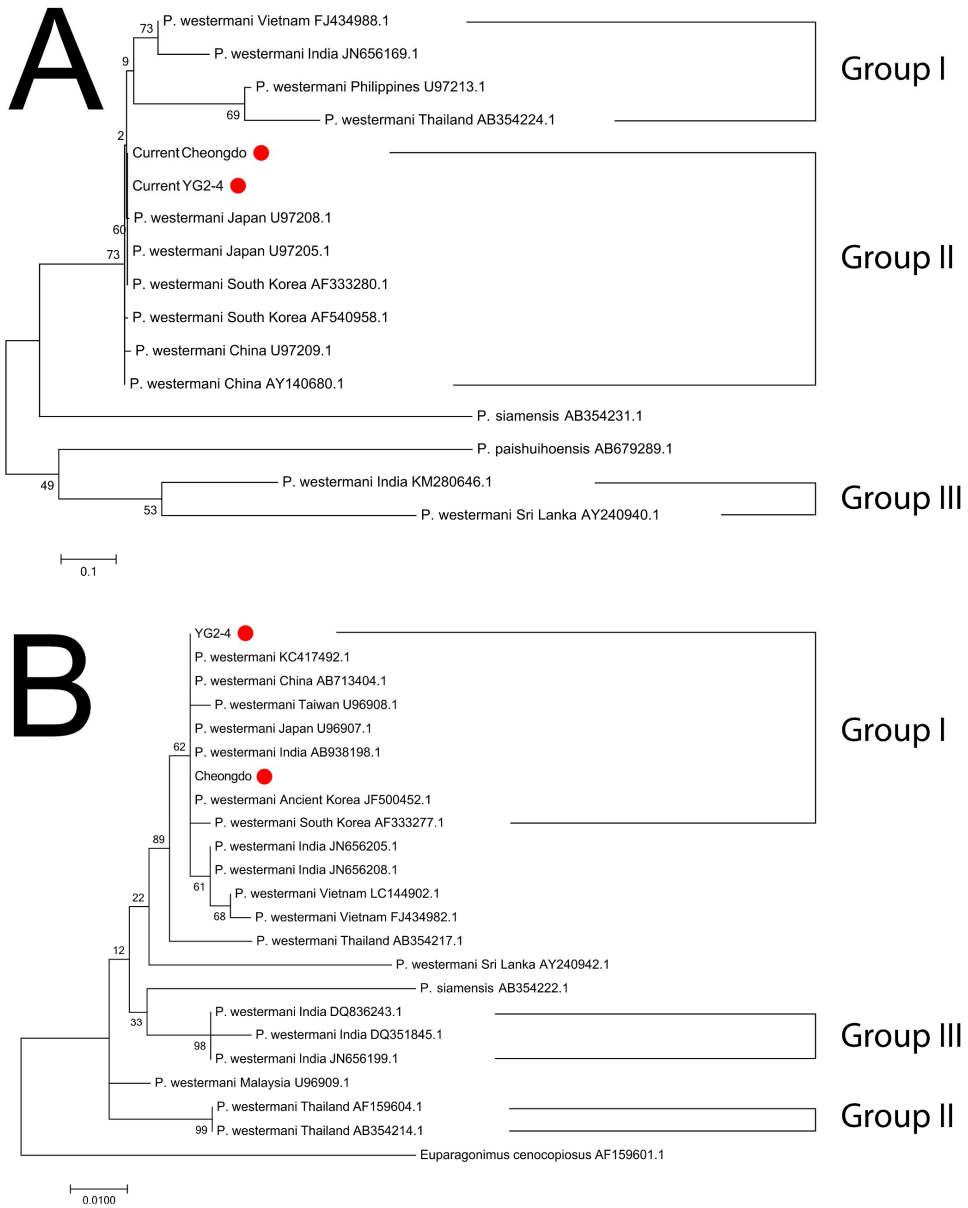

Figure 13. Maximum likelihood tree of *Paragonimus* (A) CO1 and (B) ITS2 DNA region. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are marked next to the branches. The *P. westermani* aDNA sequences revealed in this study are represented by red dots.

CHAPTER 4

Cytochrome c oxidase subunit 1, internal transcribed spacer 1, nicotinamide adenine dinucleotide hydrogen dehydrogenase subunits 2 and 5 of *Clonorchis sinensis* ancient DNA retrieved from Joseon Dynasty mummy specimens

Introduction

Clonorchis sinensis (*C. sinensis*) infects approximately 35 million people worldwide, causing various subclinical or clinical signs known as clonorchiasis (Park and Yong, 2001; Waewkes, 2003; Lun et al., 2005; Keiser and Utzinger, 2005; Sun et al., 2013). People is infected by an ingestion of undercooked or raw freshwater fish harboring metacercariae of *C. sinensis* (Kang et al., 2001; Yu et al., 2003). In a historical context, *C. sinensis* infection was one of the most common trematode infections in Korea, especially due to the cuisine based on raw fish, which was enjoyed by the inhabitants of the country (Seo et al., 2008; Ki et al., 2014).

To reveal the genetic characteristics of *C. sinensis*, researchers have attempted DNA analysis. Recently, parasitologists diagnosed *C. sinensis* through DNA analysis on internal transcribed spacer (ITS: Lee and Huh, 2004; Liu et al., 2007; Xiao et al., 2013; Tatonova et al., 2017), cytochrome c oxidase subunit (CO: Lee and Huh, 2004; Sun et al., 2013), and nicotinamide adenine dinucleotide hydrogen dehydrogenase (NAD) subunits (Sun et al., 2013; Xiao et al., 2013). The molecular analyses claimed that *C. sinensis* is genetically distinct from other trematodes (Park and Yong, 2001; Lee and Huh, 2004; Liu et al., 2007; Park, 2008; Xiao et al., 2013;

Tatonova et al., 2017).

Meanwhile, paleoparasitologists have also tried to reveal the genetic characteristics of *C. sinensis* through researches on the samples collected at archeological sites. One of such studies was carried out in South Korea. Shin et al. (2013) successfully analyzed aDNA sequences of *C. sinensis* eggs collected from the 17th century Korean mummy feces. They showed that amplified sequences of *C. sinensis* ITS1, ITS2, and CO1 were completely identical to modern *C. sinensis* sequences in GenBank (Shin et al., 2013).

Although this pioneering work was to reveal genetic traits of *C. sinensis*, the number of aDNA cases reported so far was too insufficient to get detailed information of *C. sinensis* genetics. Fortunately, by paleoparasitological studies in South Korea over the past several years, I collected a number of pre-modern Korean mummy feces or tissue specimens in which the presence of *Clonorchis* eggs was microscopically confirmed (Seo et al., 2014, 2017). Utilizing the ancient specimens, in this study, I tried to analyze CO1, ITS1, NAD2 and NAD5 of *C. sinensis* aDNA. The current report could expand the spatiotemporal scope of parasitological research about the genetic history of *C. sinensis*.

Materials and Methods

The samples used in this study were obtained from the 15th to 18th century Joseon mummies (n=5; Mungyeong, Dalsung, Cheongdo, Hadong1 and Andong) (Figure 14). The specimens were coprolites retrieved from mummy intestines (Dalsung, Hadong1 and Andong) or mummified livers (Mungyeong and Cheongdo). I followed the *Criteria of Authentication* for authentic aDNA analysis (Hofreiter et al., 2001).

For aDNA extraction, I followed the method in our previous report (Kim et al., 2011). The specimens (0.3g) were treated in a lysis buffer (1 ml) for 24 hrs at 56℃. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and then chloroform/isoamyl alcohol (24:1). DNA isolation/purification was performed by a QIAmp PCR purification kit (Qiagen, Hilden, Germany). Extract DNA (10 μl) was treated with 1 unit of uracil-DNA-glycosylase (New England Biolabs, Ipswich, USA) for 30 min at 37℃. It (40 ng) was then mixed with a reagent premix containing 10 pmol of each primer (Table 6) and 1X AmpliTaq Gold® 360 Master Mix (Life Technologies, Camarillo, USA). PCR conditions were as follows: pre-denaturation at 95 ℃ for 10 min; 45 cycles of denaturation at 95℃ for 30 sec, annealing at 54-63℃ for 30 sec,

extension at 72℃ for 30 sec, and final extension at 72℃ for 10 min. The amplified PCR products separated on 2.5% agarose gel (Invitrogen, USA) were stained by ethidium bromide. Electrophoresis also included negative (extraction) controls.

The PCR amplicon was isolated by a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Bacterial transformation was done using a pGEM-T Easy Vector system (Promega Corporation, Madison, USA). Transformed bacteria were then grown on agar plate containing X-GAL (40 μg/μl), ampicillin (50 μg/ml) and 0.5 mM IPTG for 14 hrs. After colonies were grown in LB media for 12 hrs, the cultured bacteria were purified by a QIAprep® Spin Miniprep kit (Qiagen, Hilden, Germany). Each amplified DNA strand was sequenced by an ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Waltham, USA) and 3730xl Automatic Sequencer (Applied Biosystems, Waltham, USA).

To obtain consensus sequence, multiple sequence alignment was performed for each aDNA region by Clustal W implemented in MEGA7 (Kumar et al., 2016). I compared the consensus sequences of the current *C. sinensis* to GenBank taxa by NCBI/BLAST tools (Altschul et al., 1997). The evolutionary relationship of *C. sinensis* and other parasites of NCBI GenBank was inferred by the

Phylogeny Reconstruction analysis implemented in MEGA7. I used *Maximum Likelihood* (ML) method. Selected parameters are Tamura 3-parameter (CO1; Tamura, 1992), Kimura 2-parameter (ITS1; Kimura, 1980), Hasegawa-Kishino-Yano model (NAD2 and 5; Hasegawa et al., 1985) for Model/Method. I performed bootstrap test to estimate the reliability of the tree. The number of bootstrap replicates was 500 (Hall, 2013).

Table 6. List of primers used for the amplification of *C. sinensis* DNA in this study

DNA region	Set	Primer	Sequence (5' to 3')	Annealing Temp.(℃)	Length (bp)
CO1	CO1	CO1 F	GTG TTA ATA TTG CCG GGG TTT GG	54	207
		CO1 R	ACC TAT AAT CAT AGT AAC CG		
ITS1	ITS1-2	ITS1 F2	CTG GCA CGT GTA CCC AAT A	56	122
		ITS1 R2	TCA CCC CCA ATA TGG ACT		
	ITS1-3	ITS1 F3	TCG GTA TGC TCG CTT CCG TTG	62	151
		ITS1 R3	CGG TTT GAA ATG AAC AAC AA		
	ITS1-4	ITS1 F4	GAG TGG GCA TGA TGT GTC TC	63	215
		ITS1 R4	GGC GTT ATC AGT CGT ACC CGG		
NAD2	NAD2-1	NAD2 F1	GCT ATG TTG TTG TTT CTG GTG	56	194
		NAD2 R1	ACG ACC TCT TCA AAA TGG TT		
	NAD2-2	NAD2 F2	TGA AGT TTG GTC TTT TTC CA	54	260
		NAD2 R2	TGA TGC ACT GGA ACT AAT CA		
	NAD2-3	NAD2 F3	TGG GGG TTT AAC GTT TAT TT	56	195
		NAD2 R3	CTC AGC AAC ATA ACC ACC AT		
	NAD2-4	NAD2 F4	GAG CTT TCT CCT GAT TTG CT	56	164
		NAD2 R4	ATG GAT AAA GAC CCT GGA AA		
	NAD2-5	NAD2 F5	CCG CAG TTG GGA TAT ATT TT	54	159
		NAD2 R5	ATA AAA CTG CTC CGA AAT GC		
NAD5	NAD5-1	NAD5 F1	GAT GCC GTC CTT GAT ATT TT	54	164
		NAD5 R1	CCC AAT TCT GAA AAT GAC CT		
	NAD5-2	NAD5 F2	TGC TAA ACC TCG GAG TAT GC	58	191
		NAD5 R2	CCA CCA ACC AGG AAA TAA AT		
	NAD5-3	NAD5 F3	CAG AAT TGG GTT GGT ATG TG	56	200
		NAD5 R3	CCC CTG ATA GCA GAA TAA CG		
	NAD5-4	NAD5 F4	CCC CAG TTA GTT GTT TGG TT	56	211
		NAD5 R4	GCA ACA TTT TTG CAG GTA GA		

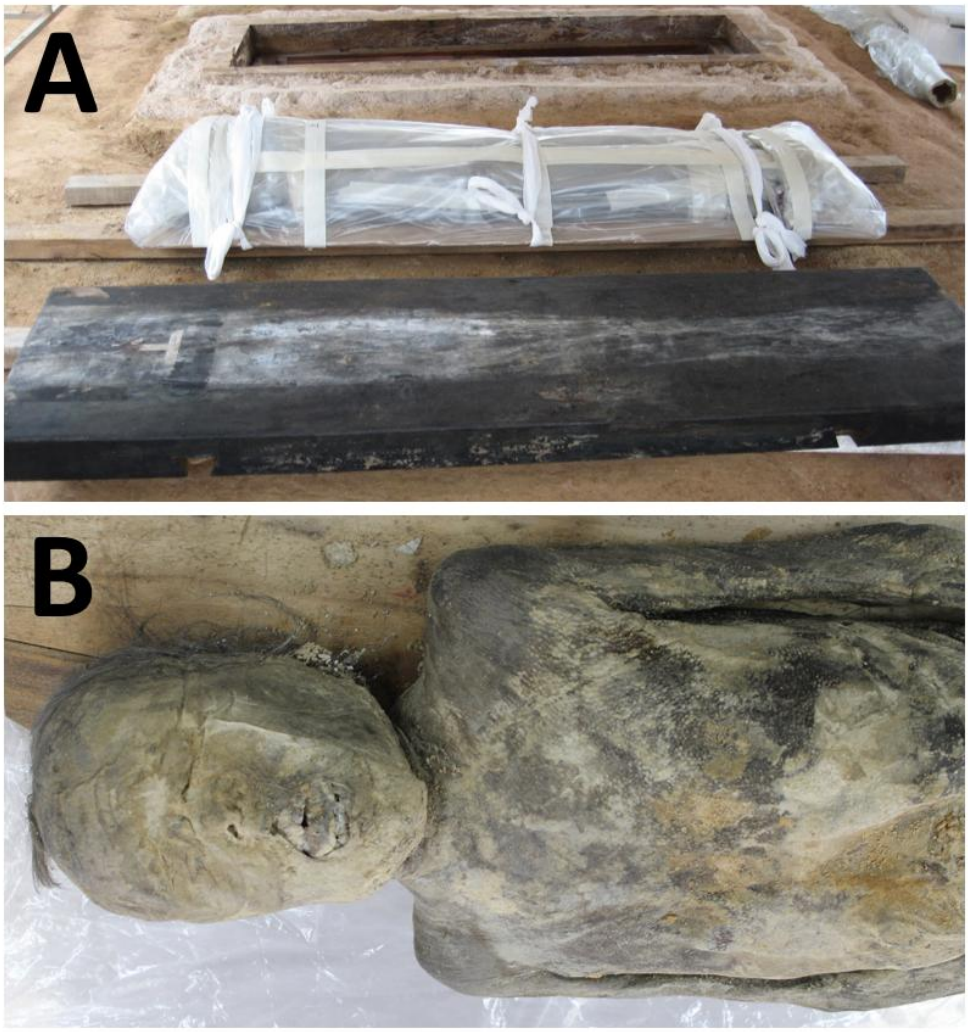

Figure 14. (A) The tomb of Joseon period. (B) A mummy (Andong) used in this study.

Results and Discussion

To select the specimens used for aDNA analysis, I screened all the mummy coprolite samples using PCR with *C. sinensis* primers for CO1 (206bp), ITS1-2 (122bp), NAD2-1 (194bp), and NAD5-1 (164bp). In agarose gel electrophoresis, negative (extraction) controls exhibited no amplified bands. In Andong feces, the PCR products were detected for *C. sinensis* CO1, ITS1-2, NAD2-1, and NAD5-1. Mungyeong specimen also showed positive results for *C. sinensis* CO1 and ITS1-2 (Figure 15). I thus used the Mungyeong and Andong specimens for subsequent aDNA analysis.

To get the consensus aDNA sequences of *C. sinensis* CO1, ITS1, NAD2, and NAD5, I tried to do cloning and sequencing of each specific amplicon. By these trials, 9-10 clone sequences were successfully acquired for CO1, ITS1, NAD2, and NAD5 amplicons (Figure 16). The total sizes of consensus sequences obtained by multiple sequence alignment were 162bp (CO1), 431bp (ITS1), 588bp (NAD2), and 443bp (NAD5), respectively. The *C. sinensis* consensus sequences of Andong and Mungyeong specimens were almost the same to each other, except for a little difference at a nucleotide position (transversions occurred in the positions CO1: 100 and ITS1: 167) (Figure 17). Considering these results, I

conjecture that genetic characteristics of *C. sinensis* might not have been uniform during the Joseon period.

In BLAST searching, the *C. sinensis* consensus sequences of Andong and Mungyeong specimens were completely or almost identical to *C. sinensis* CO1, ITS1, NAD2 and NAD 5 sequences reported in GenBank (Figure 17). Briefly, the current Joseon *C. sinensis* CO1 sequences were 100% identical to GenBank sequences of *C. sinensis* reported from South Korea (KY564177.1), China (FJ965391.1; FJ965383.1), Russia (MF406205.1; MF406204.1), and Vietnam (MF287785.1; KJ204609.1) (Table 7). *C. sinensis* ITS1 sequences of Korean mummies also exhibited very high similarities (99%) to the GenBank ITS1 sequences reported from Korea (JN638318.1; JN638320.1), China (KJ137226.1; KF740425.1; HQ186255), Russia (JQ048578.1; KC987517.1) and Vietnam (MF319655.1; MF319650.1). The aDNA sequences of Andong and Mungyeong specimens were also completely or almost (99%) identical to GenBank *C. sinensis* NAD2 and NAD5 sequences from Korea (NAD2, JF729304.1; NAD5, FJ729304.1), China (NAD2, KC170192.1; NAD5, KY564177.1), Russia (NAD2, FJ381664.2; NAD5, FJ381664.1) and Vietnam (NAD2, AY264851.1) (Table 7; Figure 17).

In the analyses, I found that CO1 region could not be an

effective marker for differential diagnosis between *C. sinensis* and other trematode species because the CO1 sequences of *Pygidiopsis summa* (AF184884.3) and *Trichinella spiralis* (AF182302.1) were not distinguishable from *C. sinensis* CO1 sequences. Meanwhile, *C. sinensis* ITS1, NAD2, and NAD5 sequences were clearly distinct from those of other trematode species (Table 7; Figure 17). I identified similar patterns in phylogenetic analyses (Figure 18). In case of CO1, ancient Andong and Mungyeong sequences belonged to the clade not only with *C. sinensis*, but also with *P. summa* and *M. xanthosomus*. On the other hand, ITS1, NAD2, and NAD5 of *C. sinensis* and other trematode species were separately clustered into different clades (Figure 18). I confirmed the usefulness of ITS1, NAD2, and NAD5 as molecular markers for differential diagnosis of *C. sinensis* from other trematode species.

In summary, my present study about *C. sinensis* aDNA retrieved from Korean mummies is designed to uncover invaluable genetic information of *C. sinensis* prevalent among pre-20th century Korean people. Although detailed understanding of *C. sinensis* genetics require a future retrieval of ancient or modern DNA sequences in wider geo-historical scope, my current report represent a significant step to improve our knowledge about genetic history of *C. sinensis*.

Table 7. BLAST searching results indicate coverage and percent identity of each taxon comparing to consensus sequence of *C. sinensis* CO1, ITS1, NAD2, and NAD5 obtained from Andong mummy. GenBank accession numbers and geographical information are also marked.

DNA region	Species	Coverage	Percent Identity	Accession Number	Geographical region
CO1	*C. sinensis*	100%	100%	KY564177.1	Korea
		100%	100%	MF287785.1	Vietnam
		100%	100%	MF406205.1	Russia
		100%	100%	FJ965391.1	China
		100%	100%	EF688130.1	Japan
		100%	99%	FJ654383.1	China
		100%	99%	KJ204609.1	Vietnam
		100%	99%	JX040566.1	Russia
		100%	99%	JF729304.1	Korea
		100%	98%	MF406206.1	Russia
		100%	97%	AF188122.2	China
		100%	96%	AF184619.2	China
	P. summa	100%	100%	AF181884.3	Korea
	T. spiralis	98%	100%	AF182302.1	unknown
	O. viverrini	100%	95%	AY055380.1	Laos
	M. xanthosomus	96%	93%	FJ423740.1	unknown
ITS1	*C. sinensis*	100%	100%	JN638318.1	Korea
		100%	100%	MF319655.1	Vietnam
		100%	100%	KJ137226.1	China
		100%	100%	JQ048578.1	Russia
		100	99%	JN638320.1	Korea
		100%	99%	KF740425.1	China
		100%	99%	MF319650.1	Vietnam
		100%	99%	KC987517.1	Russia
		100%	98%	MF319653.1	Vietnam
		100%	98%	HQ186255.1	China
	M. bilis	95%	93%	KY356536.1	Russia
	O. felineus	95%	93%	DQ456831.1	Russia
	O. veverrini	94%	91%	KX378012.1	Vietnam
NAD2	*C. sinensis*	100%	99%	JK729304.1	Korea
		100%	99%	KC170213.1	China
		100%	99%	FJ381664.2	Russia
		100%	98%	AY264851.1	Vietnam
	O. sudarikovi	100%	77%	MK033132.1	Pakistan

	M. orientalis	100%	76%	KT239342.1	China
	O. felineus	100%	76%	EU921260.2	Russia
NAD5	*C. sinensis*	100%	100%	JF729304.1	Korea
		100%	99%	KY564177.1	China
		100%	99%	FJ381664.2	Russia
	O. sudarikovi	99%	81%	MK033132.1	Pakistan
	M. orientalis	99%	81%	KT239342.1	China
	O. felineus	98%	80%	EU921260.2	Russia

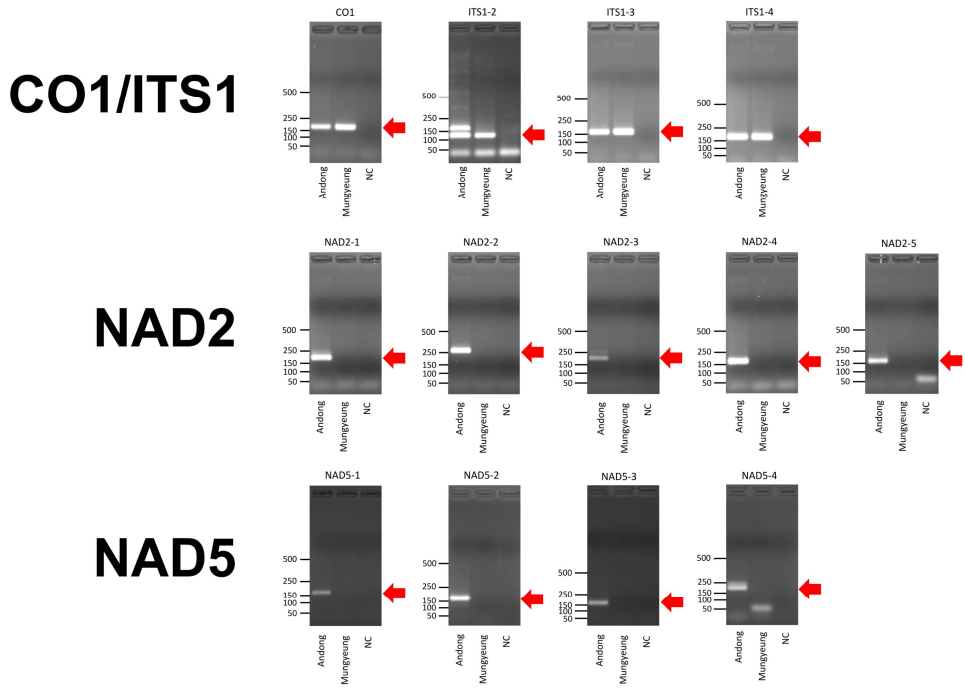

Figure 15. Agarose gel electrophoresis of the PCR products amplified from Joseon *C. sinensis* samples. Specific bands were indicated by arrows. NC, negative control.

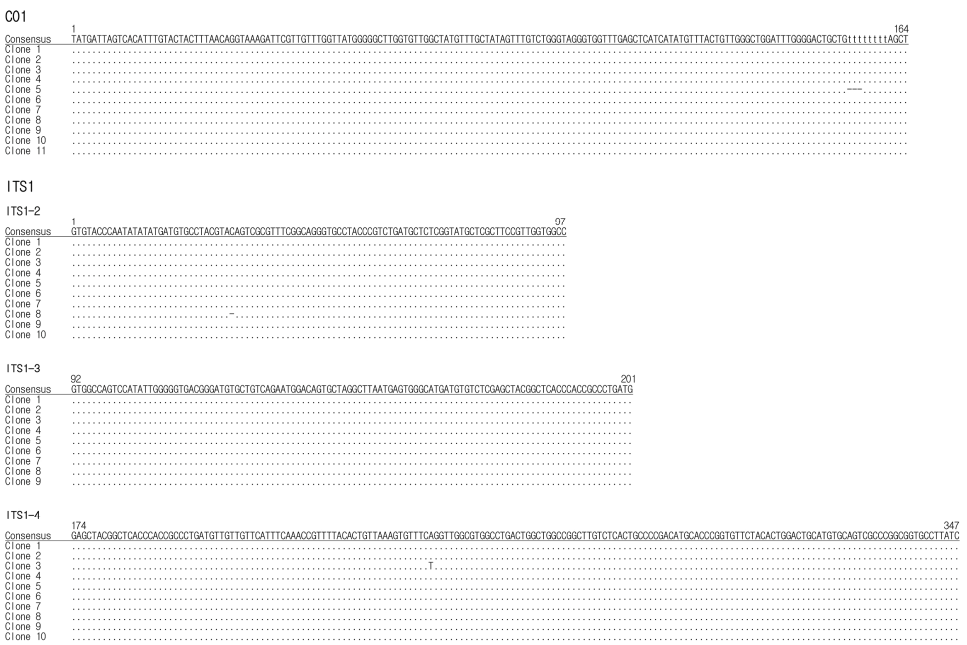

Figure 16-1. Aligned clone sequences of CO1, ITS1, NAD2, and 5 DNA fragments from Joseon Dynasty mummies. (A) Andong mummy.

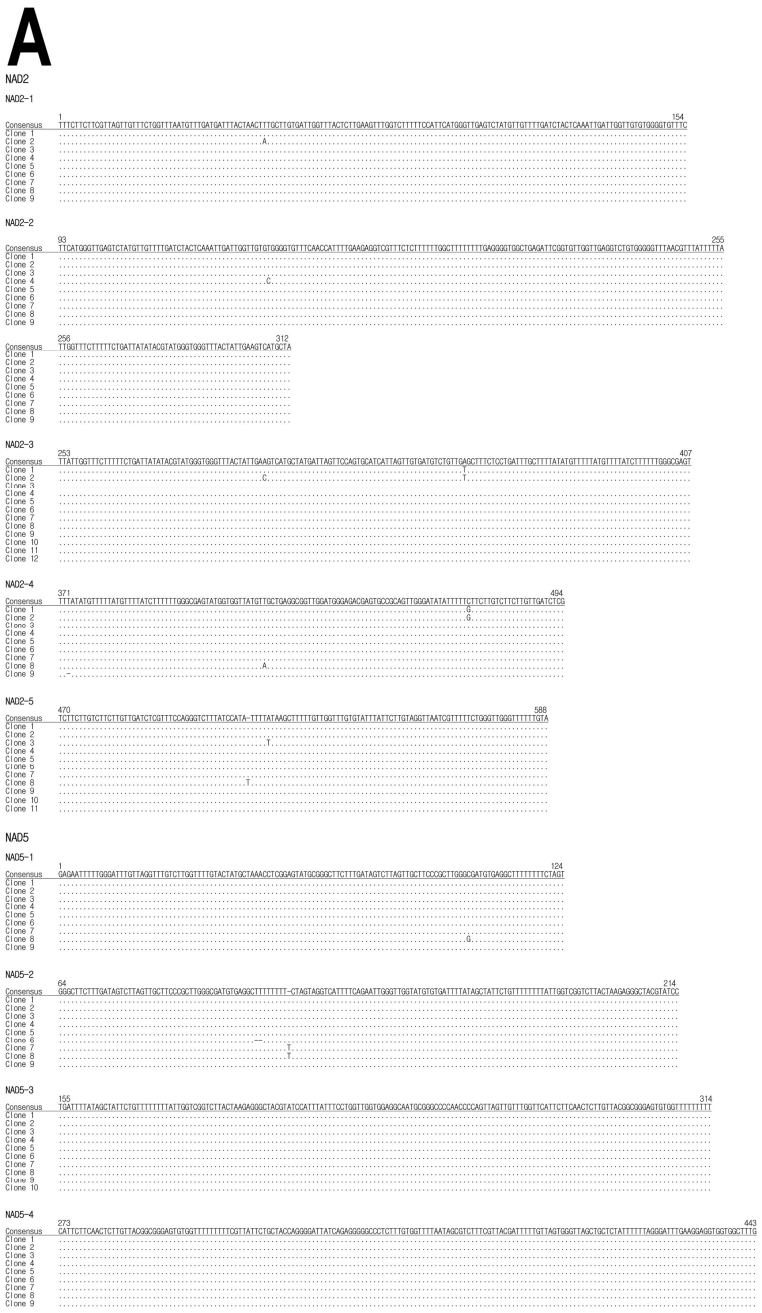

Figure 16-2. Aligned clone sequences of CO1, ITS1, NAD2, and 5 DNA fragments from Joseon Dynasty mummies. (A) Andong mummy.

Figure 16-3. Aligned clone sequences of CO1 and ITS1 DNA fragments from Joseon Dynasty mummies. (B) Mungyeong mummy.

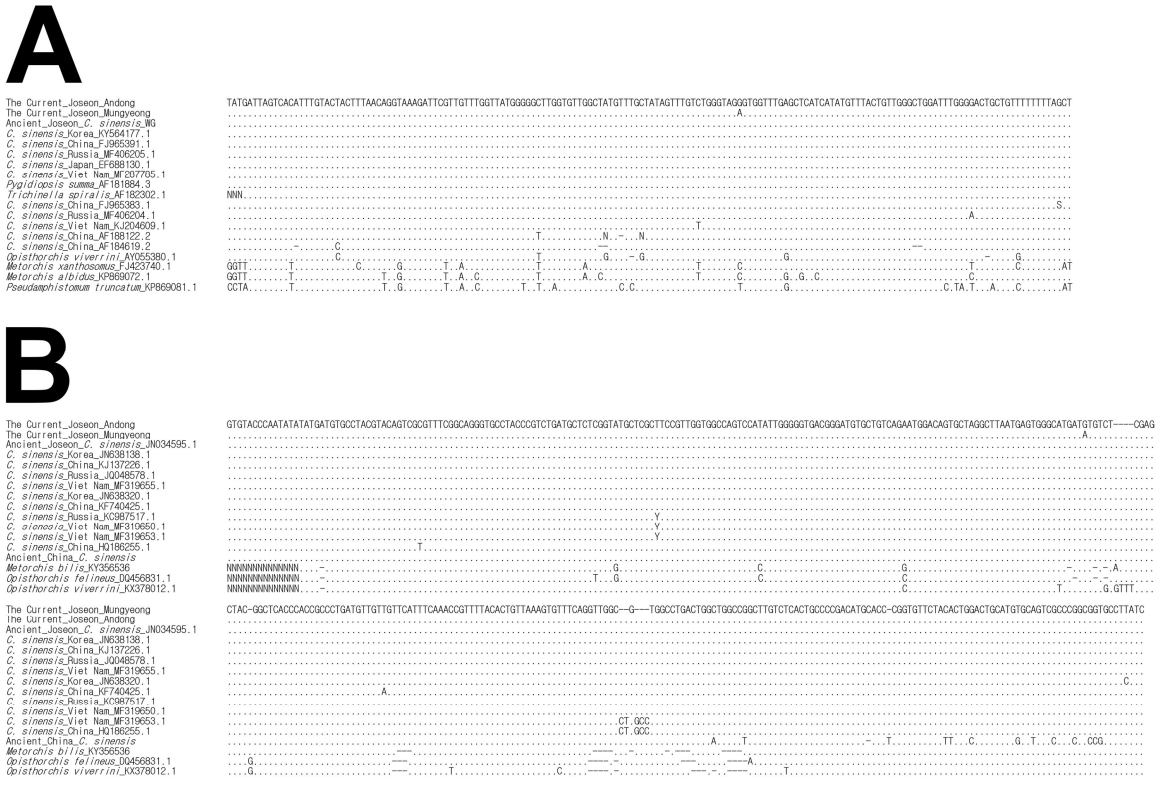

Figure 17-1. BLAST analyses of the consensus DNA sequences from *C. sinensis* and other sequences in GenBank. (A) CO1, (B) ITS1, (C) NAD2, and (D) NAD5 DNA regions.

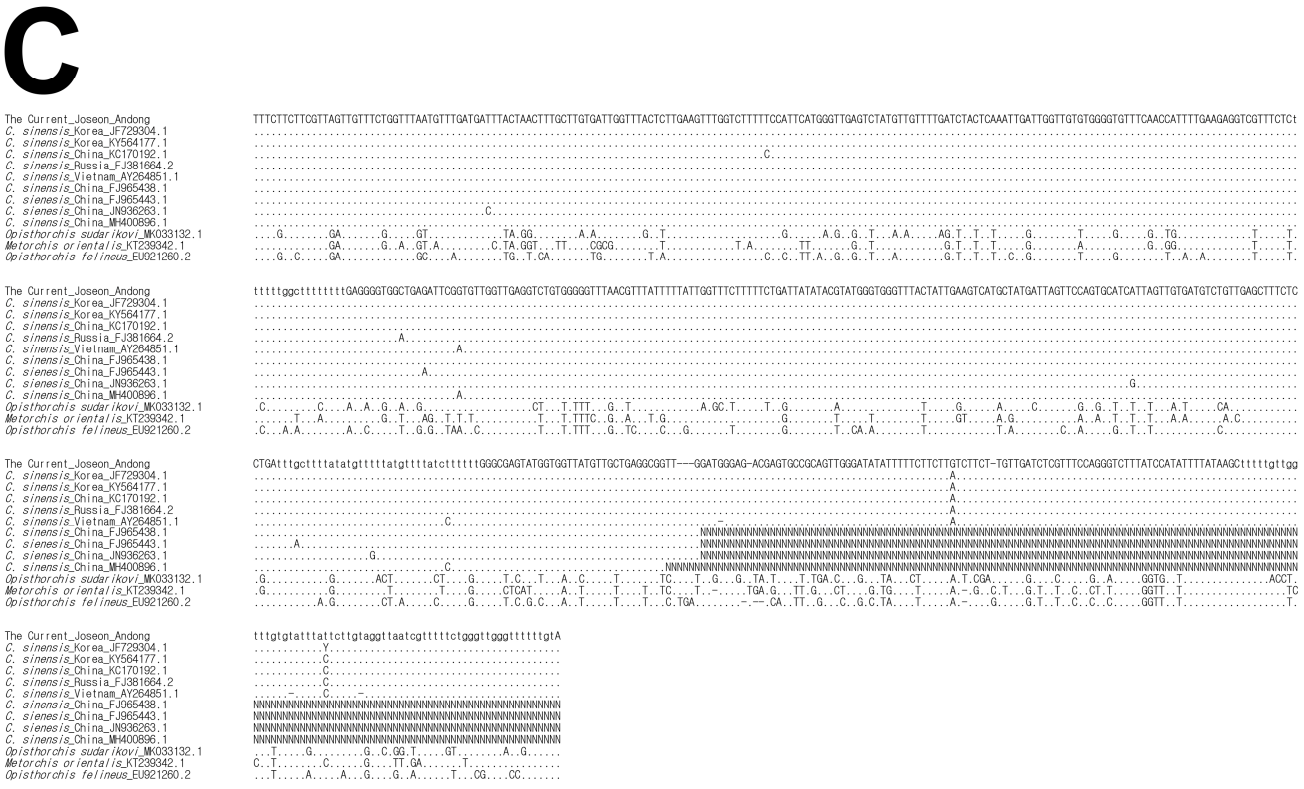

Figure 17-2. BLAST analyses of the consensus DNA sequences from *C. sinensis* and other sequences in GenBank. (A) CO1, (B) ITS1, (C) NAD2, and (D) NAD5 DNA regions.

D

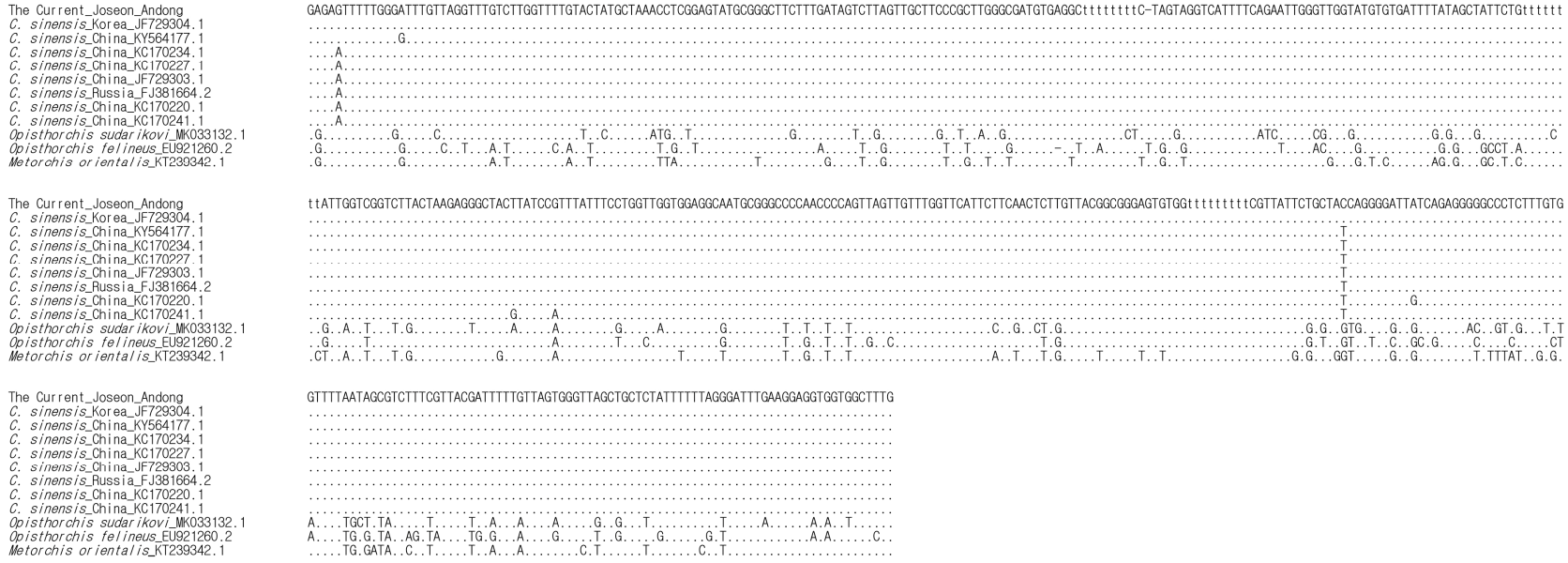

Figure 17-3. BLAST analyses of the consensus DNA sequences from *C. sinensis* and other sequences in GenBank. (A) CO1, (B) ITS1, (C) NAD2, and (D) NAD5 DNA regions.

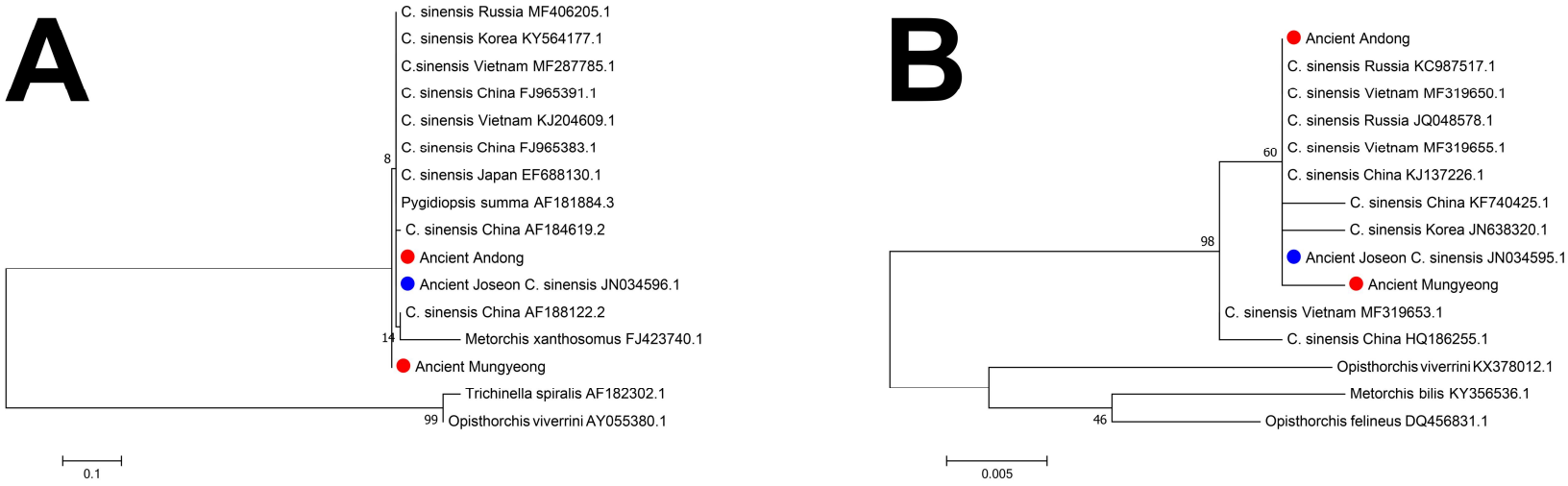

Figure 18-1. Phylogenetic analyses (ML method) of the current *C. sinensis* (red dots) and the other trematodes in GenBank. (A) CO1, (B) ITS1, (C) NAD2, and (D) NAD5 DNA regions. Blue dots, Joseon *C. sinensis* sequence previously reported by Shin et al. (2013).

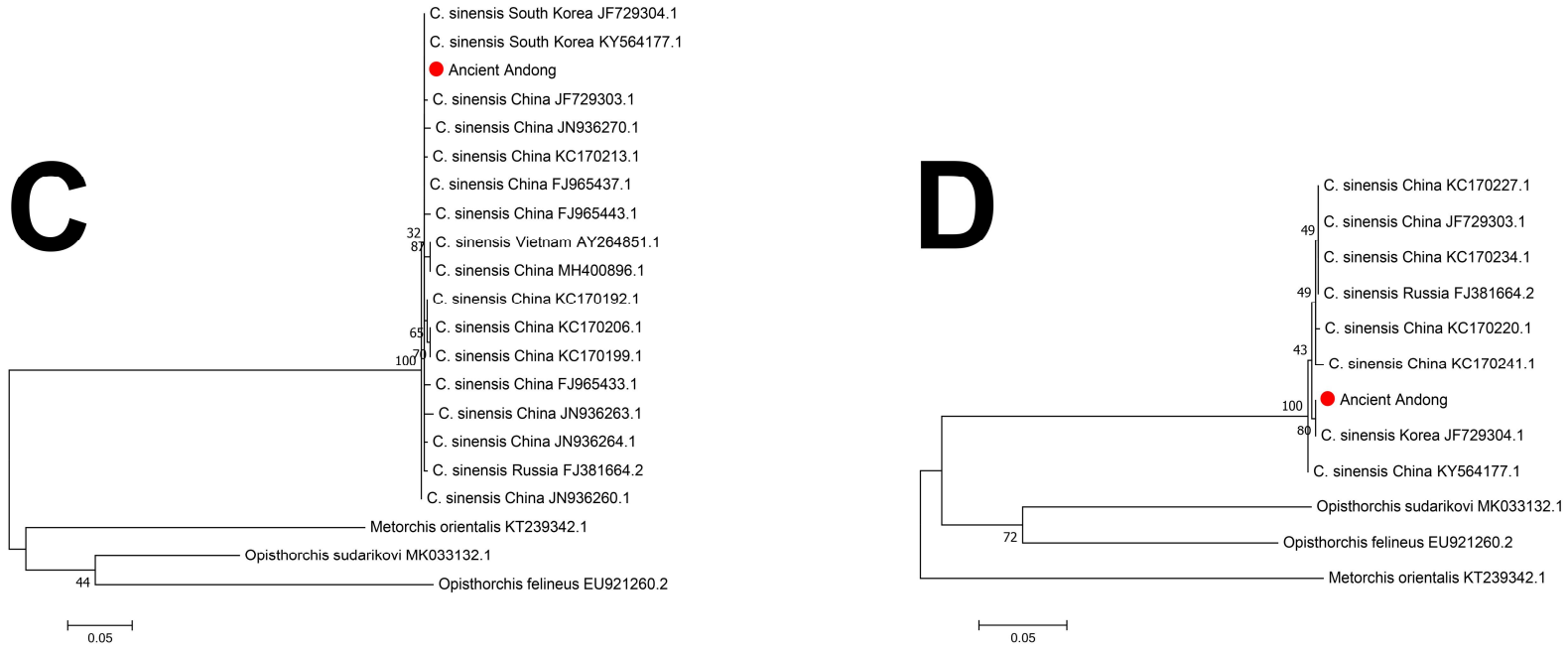

Figure 18-2. Phylogenetic analyses (ML method) of the current *C. sinensis* (red dots) and the other trematodes in GenBank. (A) CO1, (B) ITS1, (C) NAD2, and (D) NAD5 DNA regions. Blue dots, Joseon *C. sinensis* sequence previously reported by Shin et al. (2013)

CHAPTER 5

Metagonimus yokogawai ancient DNA recovered from the 16th to 17th century Korean mummy feces of Joseon Dynasty

Introduction

Metagonimiasis is one of the major foodborne intestinal parasite infection occurring in definitive hosts by their ingestion of raw or undercooked sweetfish etc. (Ito, 1964; Chai et al., 2009; Shimazu and Kino, 2015). Human infections of *Metagonimus* spp. were reported from different countries such as Korea, Japan, China, Taiwan, Far East provinces of Russia, Israel, the Balkan states, and Spain (Yu and Mott, 1994; Chai et al., 2009; Pottinger and Jong, 2016).

Metagonimus infection is most commonly caused by *M. yokagawai* (Katsurada, 1912; Lee et al., 2004; Chai et al., 2009), but also rarely by *M. takahashii* (Suzuki, 1930) as well as *M. miyatai* (Miyata, 1941; Saito et al., 1997; Chai et al., 2009). These parasite species of genus *Metagonimus* could be differentiated by morphology (Chai et al., 1991, 1998, 2000), intermediate host (Rim et al., 1996), infecting organs in the host (Kino et al., 2006), and geographical distribution (Kim et al., 1987; Chai et al., 1993; Yu et al., 1994; Lee et al., 2002).

In addition to such analyses, genetic techniques have been also carried out to differentiate the species of genus *Metagonimus*. In previous studies of random amplification of polymorphic DNA

(RAPD) and polymerase chain reaction based-restriction fragment length polymorphism (PCR-RFLP), *M. takahashii* and *M. miyatai* were genetically distinct from *M. yokogawai* (Yu et al., 1997a, 1997b; Chai et al., 2009). Sequencing and phylogenetic analysis of 28S ribosomal DNA (rDNA) and cytochrome c oxidase subunit I (CO1) were also performed for *M. yokogawai*, *M. takahashii*, and *M. miyatai* (Yu et al., 1997; Lee et al., 2004).

Despite such pioneering researches, there have been no studies on the genetic characteristics of *Metagonimus* DNA inferred from archaeological specimens worldwide. As the aDNA analysis is an invaluable method for conjecturing the genetic history of *M. yokogawai* in spatiotemporal perspective, I need to analyze *Metagonimus* aDNA using much more ancient coprolite samples obtained from archaeological sites. Fortunately, in our paleoparasitological studies, I found *M. yokogawai* eggs in 3 of 24 coprolites of the 16th- to 17th-century Korean mummy coprolites (Seo et al., 2008, 2014, 2017). Using the *M. yokogawai* eggs in coprolites, I tried to perform genetic analysis on 28S rDNA and CO1 of *M. yokogawai* to get fundamental information on the genetic history of genus *Metagonimus* spp.

Materials and Methods

The samples used in this study were ancient coprolites obtained from the 16th to 17th century Korean mummies (HD1, Sapgyo, and Sacheon) of Joseon Dynasty, in which *M. yokogawai* eggs were identified by microscopic examination (Seo et al., 2008, 2014; Shin et al., 2012). The geo-archaeological information of Korean mummies used in this study are summarized in Figure 19 and Table 8.

For authentic aDNA analysis, I followed the *Criteria of Authentication* by Hofreiter et al. (2001). In brief, I wore protection gloves, masks, gowns and head caps during aDNA analysis. All the tools used in this study were sterilized before use. I also did experiments in specialized facility that was exclusively dedicated to aDNA analysis (Hofreiter et al., 2001; Ho and Gilbert, 2010). The Institutional Review Board (IRB) of Seoul National University Hospital admitted that my aDNA analyses could be exempt from the board review (IRB No. 2017-001). I followed the Vermillion Accord on Human Remains of World Archaeological Congress (Fforde, 2014).

To extract DNA from the specimen, I followed the method described in previous report (Kim et al., 2011). The samples (0.3

g) were incubated in 1 ml of lysis buffer (pH 8.0; including 50 mM of EDTA; 1 % SDS; 0.1 M DTT; 1 mg/ml of proteinase K) at 56℃. After DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), it was treated again with chloroform/isoamyl alcohol (24:1). DNA isolation and purification were performed using a QIAmp PCR purification kit (Qiagen, Hilden, Germany). The purified DNA was eluted in 40 μl of EB elution buffer (Qiagen, Hilden, Germany). PCR Primers for *M. yokogawai* 28S rDNA and CO1 were generated by the Integrated DNA Technologies, Inc. (Iowa City, IA, USA). The information of PCR primers is summarized in Table 9.

DNA quantification was done by NanoDrop™ ND-1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA). Extracted DNA (10 μl) was treated with 1 unit of uracil-DNA-glycosylase (New England Biolabs, MA, USA) at 37℃. It (40 ng) was then mixed with the reagent premix containing 1X AmpliTaq Gold® 360 Master Mix (Life Technologies, CA, USA) and 10 pmol of each primer. PCR conditions were as follows: pre-denaturation at 95℃ for 10 min; 45 cycles of denaturation at 95℃ for 30 sec, annealing at 52-55℃ for 30 sec, extension at 72℃ for 30 sec; and final extension at 72℃ for 10 min. The PCR products were separated on 2.5% agarose gel (Invitrogen, CA, USA), and then

stained with ethidium bromide. Negative controls (extraction controls) were also applied to the electrophoresis. The results were photographed using a Vilber Lourmat ETX-20.M equipped with Biocapt software (Vilber Lourmat, Collégien, France).

The PCR amplicons were isolated using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Bacterial transformation was done with the pGEM-T Easy Vector system (Promega Corporation, Madison, USA). Transformed bacteria were grown in agar plates containing ampicillin (50 μg/ml), X-GAL (40 μg/μl), and 0.5 mM IPTG. Selected colonies were grown in LB media for 12 hrs; and the cultured bacteria was purified by a QIAprep® Spin Miniprep kit (Qiagen, Hilden, Germany).

Each amplified strand was sequenced with an ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Waltham, USA) and the 3730xl Automatic Sequencer (Applied Biosystems, Waltham, USA). To obtain consensus sequence, clone sequences were aligned by Clustal W implemented in MEGA7 program (Kumar et al., 2016). Web browser module and Alignment Explorer implemented in MEGA7 and NCBI/BLAST tools (Altschul et al., 1997) were used for retrieving GenBank sequences homologous to the consensus sequences.

The evolutionary relationship of genus *Metagonimus* spp. and the other taxa from NCBI GenBank was inferred by the Phylogeny Reconstruction analysis implemented in MEGA7. I used Maximum Likelihood (ML) method. Selected parameters of Model/Method were as follows: Kimura 2-parameter model (Kimura, 1980) for 28S rDNA and Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) for CO1; Uniform Rates (28S rDNA) or Has Invariant sites (CO1) for Rates among Sites; Complete deletion for Gaps/Missing data treatment; and Nearest-Neighbor-Interchange (NNI) for ML Heuristic Method. To estimate the reliability of the tree, I did bootstrap test (Hall, 2013). The number of bootstrap replicates was 500.

Table 8 The archaeological information of Korean mummies in this study.

Cases	Estimated date	Sample type	sex	Year of Discovery
HD1	17C	Feces	F	2006
Sapgyo	16C	Precipitates on hip bone	M	2011
Sacheon	17C	Precipitates on hip bone	F	2011

Table 9. List of primers used for amplification of *M. yokogawai* DNA in this study.

Locus	Primer set	Sequence (5' → 3')	Annealing Temp. (°C)	PCR cycles	Amplicon size (bp)
28S rDNA	MY_28S_F1	GCT GCA TTC ACA AAC ACC CCG ACT C	55	45	114
	MY_28S_R1	CCA TGT CAG CAT TAC CGT AC			
	MY_28S_F2	CAC TGA CAT TTT TGC ACC TC	55		192
	MY_28S_R2	TAA GTA ACG GCG AGT GAA CA			
	MY_28S_F3	TGA ACA CCA CAT TGC CTA GT	52		138
	MY_28S_R3	GAT TAC CCG CTG AAC TTA AGC ATA			
CO1	MY_COI_F1	GTG TTG ATT CTC CCA GGG GTT	54		156
	MY_COI_R1	GAC CCA CCA TAA ACA TGT GAT GC			
	MY_COI_F2	TGA TTC CTT GTT TGG CTA TG	55		152
	MY_COI_R2	GCA CCC CAA TTA CCA TAG TT			
	MY_COI_F3	ACG GCA GTC TTC TTT AGC TC	55		134
	MY_COI_R3	AAC CCA AGT ATC CAC CAC AT			
	MY_COI_F4	GTA TAA AGG TGT TTT CCT GGC TGT A	55		177
	MY_COI_R4	CTA TCA CAA ACC AAG TGT CAT GCA A			

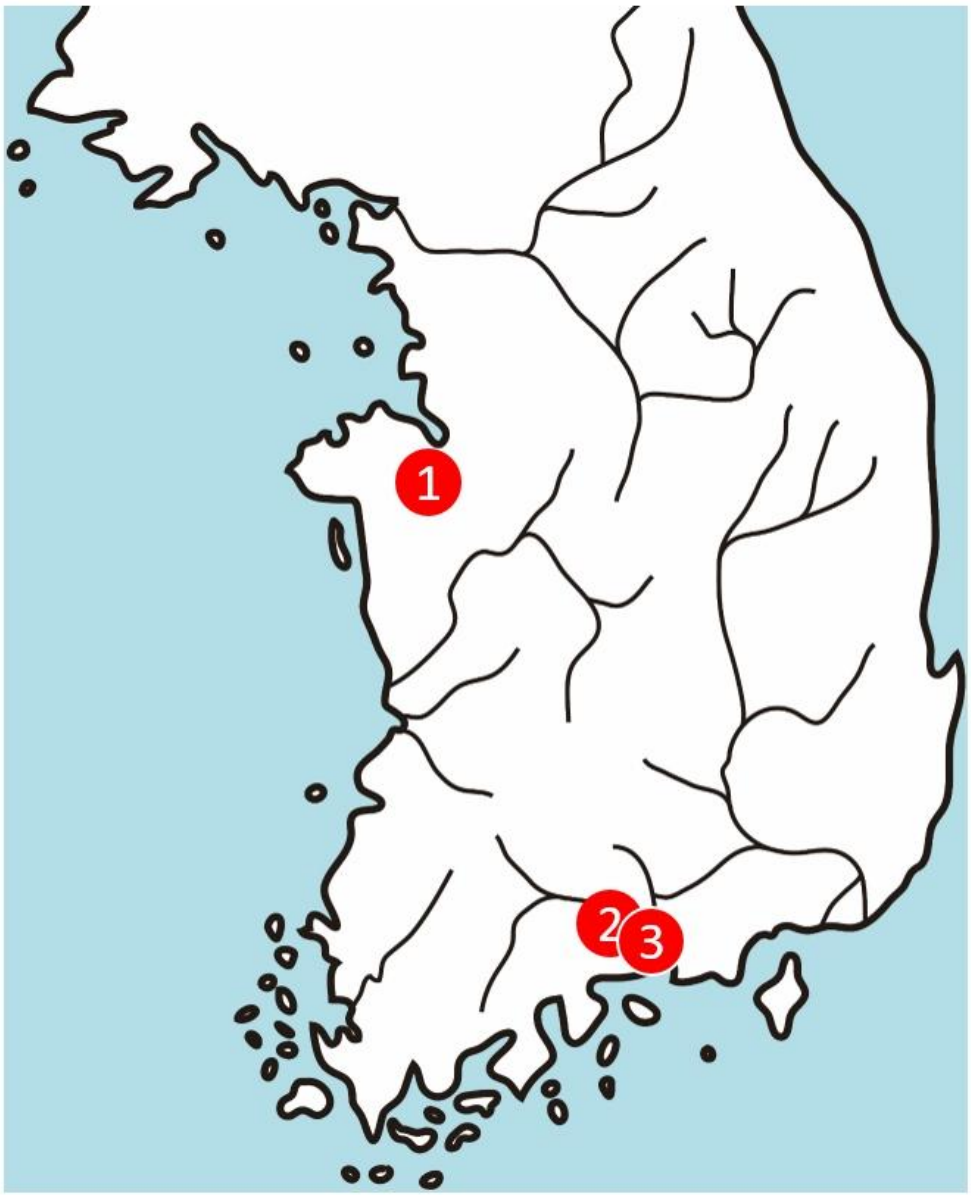

Figure 19. The map of South Korea. Red dots represent the sites where the mummies of the current studies were found. 1, Sapgyo; 2, HD1; 3, Sacheon.

Results and Discussion

In agarose gel electrophoresis, the PCR amplified products of *M. yokogawai* aDNA were detected in the specimens of Joseon mummies (HD1 and Sapgyo) while negative controls (extraction controls) did not exhibit any amplified bands (Figure 20). To get the consensus sequences of 28S rDNA and CO1, I repeated cloning and sequencing for each amplicon of specific size. By these trials, 9-11 clone sequences were successfully acquired for 28s rDNA (248-249bp) and CO1 (347bp) (Figure 21). I obtained consensus sequences by the alignment of multiple clone sequences. *M. yokogawai* 28S rDNA sequences of HD1 and Sapgyo mummies were almost the same to each other except for differences at two different nucleotide positions (transversions at position 211; insertion at position 8) (Figure 22).

By NCBI/BLAST tools, I compared the consensus sequences (HD1 and Sapgyo) to the taxa in GenBank (Figure 22). In brief, the *M. yokogawai* of Korean mummies exhibited very high similarity to GenBank sequences of *M. yokogawai* 28S rDNA reported from Korea (AF095331.2) as well as Japan (HQ832639.1). I also found similar GenBank sequences of *M. pusillus* (MF407173.1), *M. suifunensis* (KX387460.1), *M. miyatai* (AF095333.2), *M. takahashii*

(AF095332.2), *Euryhelmis costaricensis* (AB521800.1), *Clonorchis sinensis* (EF654661.1), *Paragonimus westermani* (AF095329.2) and *Pygidiopsis summa* (AF181885.3) (Figure 22A). In BLAST analysis, the *M. yokogawai* 28S rDNA sequences of Korean mummies were almost the same as GenBank sequences of *M. yokogawai, M. takahashii, M. miyatai, M. pusillus*, and *M. suifunensis* etc. This finding is well matched with the previous report of Lee et al. (2004). In their study, nucleotide sequence differences among *M. yokogawai*, *M. takahashii* and *M. miyatai* species were less than 0.8 % (2/248), making the differentiation of genus *Metagonimus* spp. very hard for the singular analysis of 28S rDNA.

On the other hand, in case of CO1, the sequence of Korean mummy (HD1) was almost identical to Genbank CO1 sequences of *M. yokogawai,* but was evidently distinct from the other parasite species. Actually, *M. yokogawai* of HD1 mummy exhibited very high similarity to the GenBank taxa of *M. yokogawai* CO1 reported from Korea (KC330755.1; AB470519.1; AF096230.3) and Japan (KM061413.1; KM061414.1). On the other hand, relatively low similarity was identified in *M. miyatai* (KM061410.1), *M. takahashii* (KM061408.1), *M. hakubaensis* (KM061416.1), *M. suifunensis* (MF406217.1), *M. katuradai* (KM061418.2), *M. pusillus* (MF406210.1), *M. otsurui* (KM061422.1) and *Ascocotyle*

pindoramensis (MF967605.1) (Figure 22B).

Our phylogenetic analysis of *M. yokogawai* 28S rDNA and CO1 re-confirmed the results of BLAST analysis (Figure 22). In phylogenetic tree of 28S rDNA, *M. yokogawai* aDNA sequences of Korean mummies (HD1 and Sapgyo) were clustered together with the other species of genus *Metagonimus* in GenBank (Figure 23A). In case of CO1 region, however, *M. yokogawai* formed a separate cluster that was evidently distinct from the other parasite species (Figure 23B). As seen in previous reports of Lee et al. (2004) and Chontananarth et al. (2014), the academic value of CO1 analysis in molecular diagnosis of *M. yokogawai* could be confirmed in this study once again though more studies on the samples representing a wide geo-historical scope are still needed to understand the genetic history of *M. yokogawai* more comprehensively.

In the present study, using the ancient coprolites retrieved from the 16th to 17th century Korean mummies, I successfully analyzed 28S rDNA and CO1 of *M. yokogawai* aDNA. I found that genetic characteristics of *M. yokogawai* of the Joseon period were not uniform because the sequences I obtained showed genetic difference at a few nucleotide positions. Our analysis confirmed the usefulness of CO1 in the molecular diagnosis of *M. yokogawai* because Korean mummy's *M. yokogawai* CO1 was clustered

together with *M. yokogawai* but was evidently distinct from *M. takahashii* and *M. miyatai* taxa in GenBank. Since the current study is the first-ever reports on *M. yokogawai* aDNA so far, it will be a fundamental basis of *M. yokogawai* paleogenetics in the future.

Table 10. BLAST analysis. Consensus sequences of *M. yokogawai* aDNA from HD1 and Sapgyo mummies were compared to the sequences retrieved at GenBank. GenBank accession numbers and geographical information are also indicated.

DNA region	Species	Coverage	Percent Identity	Accession Number	Geographical region
28S rDNA	*M. pusillus*	100%	100%	MF407173.1	Russia
		100%	100%	MF407172.1	Russia
	M. suifunensis	100%	100%	KX387460.1	Russia
		100%	100%	KX387456.1	Russia
	M. miyatai	100%	99.60%	AF095333.1	South Korea
		91%	100%	HQ832639.1	Japan
	M. takahashii	100%	99.20%	AF095332.2	South Korea
		91%	100%	HQ832636.1	Japan
	M. yokogawai	100%	98.80%	AF095331.2	South Korea
		91%	100%	HQ095331.2	Japan
	E. costaricensis	100%	97.58%	AB521800.1	Japan
	C. sinensis	100%	95.56%	EF654661.1	Unidentified
	P. westermani	100%	95.56%	AF095329.1	Unidentified
	P. summa	100%	95.56%	AF181885.3	South Korea
CO1	*M. yokogawai*	100%	99.71%	KC330755.1	South Korea
		100%	99.71%	AB470519.1	South Korea
		96%	100%	KM061413.1	Japan
		96%	100%	KM061412.1	Japan
		96%	99.70%	KM061414.1	Japan
		100%	96.55%	AF096230.3	South Korea
	M. miyatai	96%	87.24%	KM061410.1	Japan
		96%	86.94%	KM061411.1	Japan
		97%	83.43%	AF096232.3	Unidentified
	M. takahashii	96%	86.69%	KM061407.1	Japan
		96%	86.09%	KM061408.1	Japan
		99%	85.34%	AF096231.3	Unidentified
	M. hakubaensis	95%	86.79%	KM061416.1	Japan
		95%	86.49%	KM061415.1	Japan
	M. suifunensis	96%	85.76%	MF406217.1	Russia
		96%	85.46%	MF406214.1	Russia
	M. katsuradai	96%	85.50%	KM061418.2	Japan
	M. pusillus	96%	84.87%	MF406210.1	Russia
		96%	84.87%	MF406209.1	Russia
	M. otsurui	96%	84.32%	KM061422.1	Japan
		96%	84.02%	KM061421.1	Japan
	A. pindoramensis	99%	80.92%	MF967605.1	Brazil

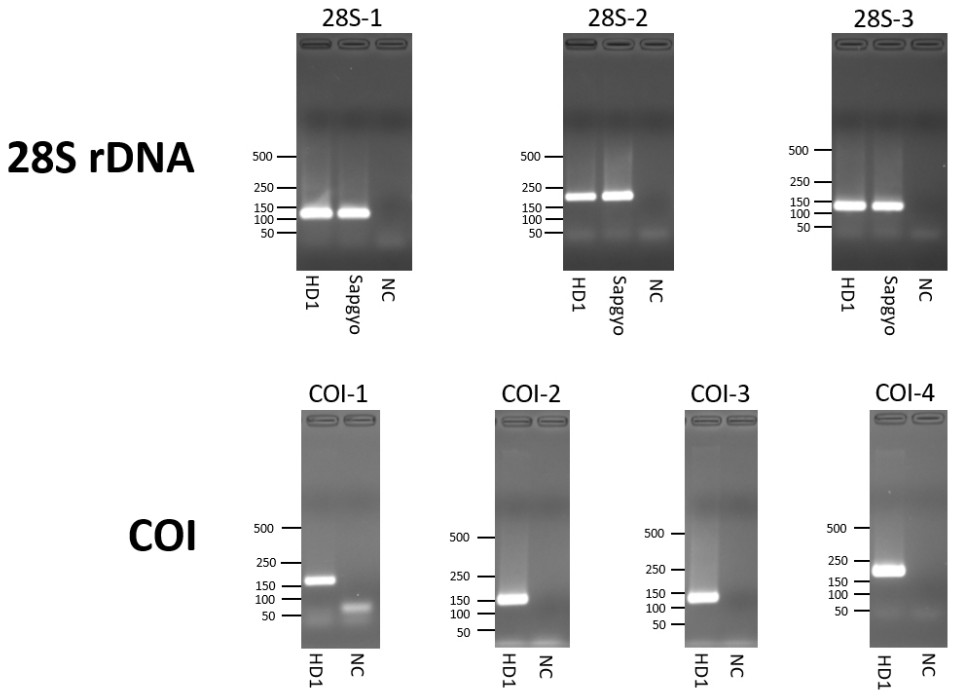

Figure 20. Agarose gel electrophoresis for the PCR amplicons of *M. yokogawai* aDNA from HD1 and Sapgyo mummies. Note specific amplicons 1 (114bp), 2 (192bp), 3 (138bp) of 28S rDNA; and 1 (156bp), 2 (152bp), 3(134bp), 4 (177bp) of CO1.

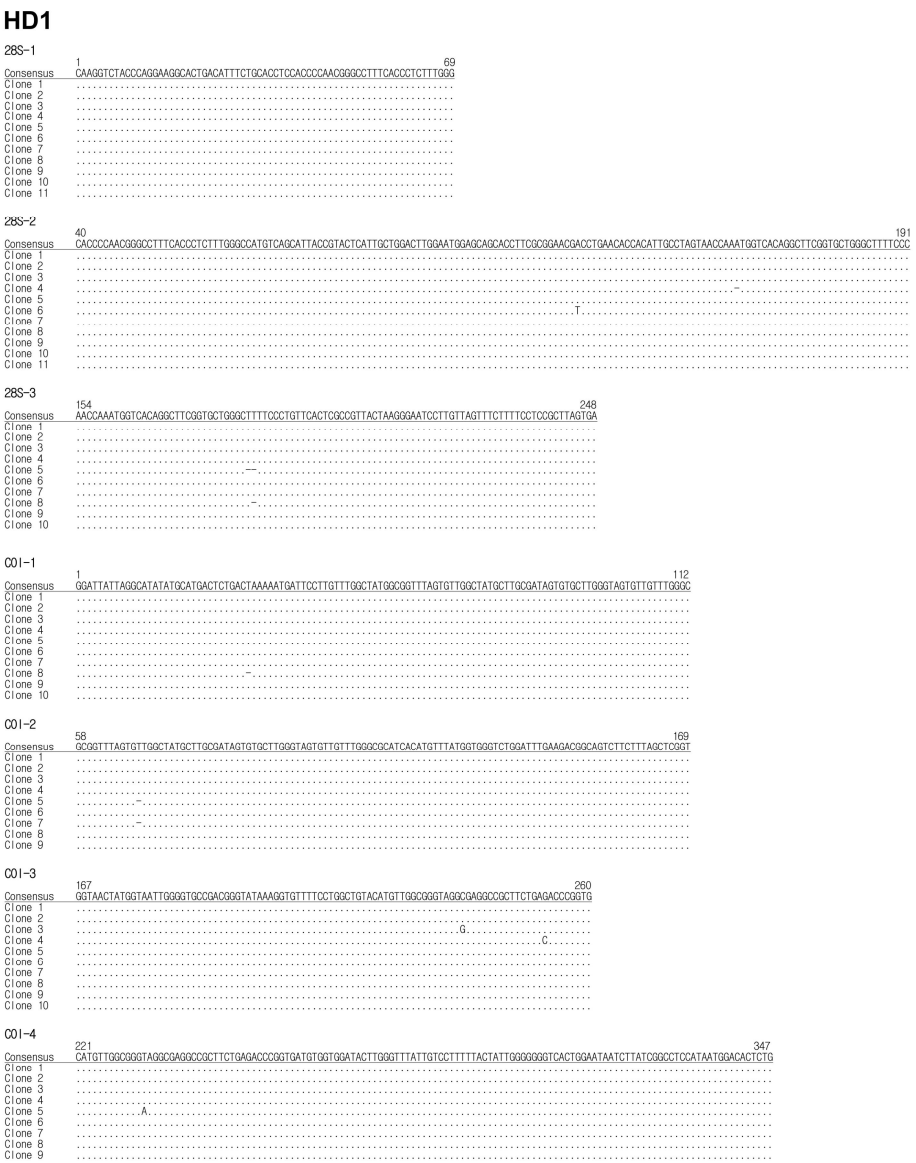

Figure 21-1. Aligned clone sequences of 28S rDNA and CO1 fragments from HD1 mummy.

Figure 21-2. Aligned clone sequences of 28S rDNA fragments from Sapgyo mummy.

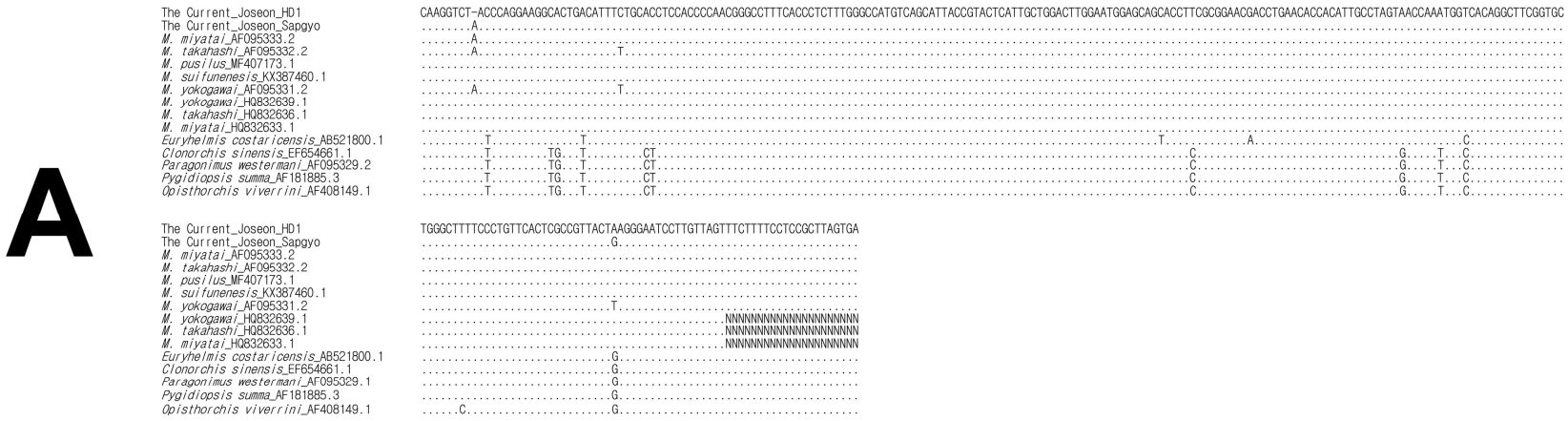

Figure 22-1. BLAST analysis. Consensus sequences of *M. yokogawai* aDNA from HD1 and Sapgyo mummies were compared to the sequences retrieved at GenBank. (A) *M. yokogawai* 28S rDNA.

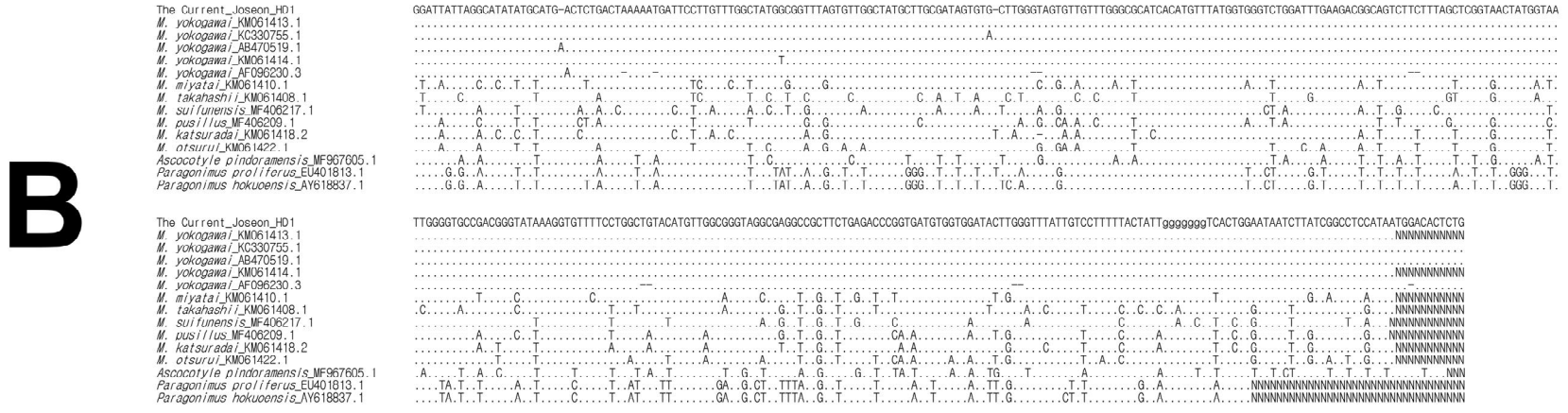

Figure 22-2. BLAST analysis. Consensus sequences of *M. yokogawai* aDNA from HD1 mummy was compared to the sequences retrieved at GenBank. (B) *M. yokogawai* CO1.

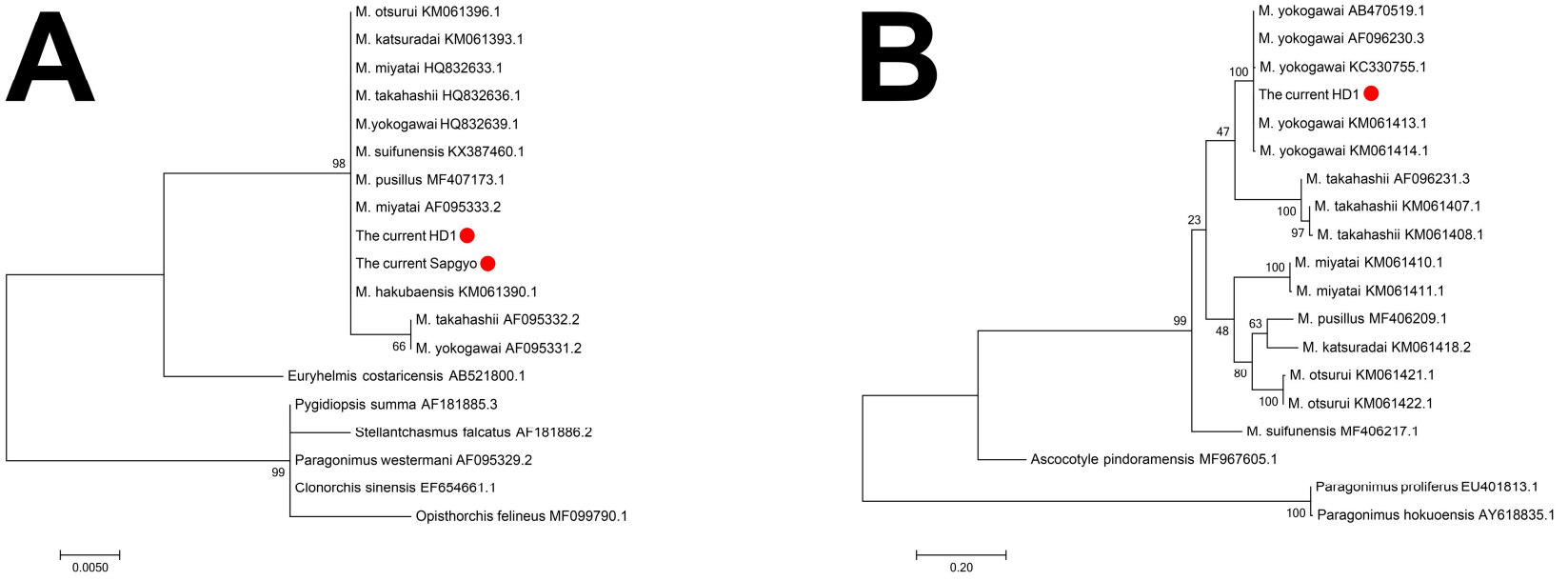

Figure 23. Phylogenetic tree for (A) 28S rDNA and (B) CO1 by Maximum Likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are marked next to the branches. The *M. yokogawai* sequences revealed in this study are represented by red dots.

Conclusion

Ancient DNA analysis is based on key techniques developed over the past few decades in the fields of molecular biology and archaeological science. In that time, aDNA study has become a significant area of interest in archeology as well as anthropology. In fact, scholars have achieved remarkable results in studies on ancient infectious diseases, and the scope of such investigations continues to expand.

Nowadays, aDNA analysis is a major field within the discipline of paleoparasitology, and it is expected to prove useful as a complementary method to resolve issues that remain in dispute after treatment by conventional techniques. However, it is true that the number of aDNA cases reported so far is insufficient to provide detailed information on parasite genetics. In this regard, my thesis will be meaningful to concerned researchers interested in aDNA analysis on paleoparasitological specimens.

In the present study, using coprolite and tissue specimens from 15th to 18th century Korean mummies, I successfully characterized multiple DNA region sequences of *Ascaris* spp., *T. trichiura*, *P. westermani*, *C. sinensis,* and *M. yokogawai*. I found that

each parasite DNA sequence belonged to separate clusters that were evidently distinct from those of the other parasite species reported to date. I thus proved the usefulness of aDNA analysis in the differential diagnosis of each parasite species, even for cases where the ancient parasite eggs found in archaeological sites were difficult to identify by microscopic examination.

The current report on the five species of parasite aDNA provides invaluable genetic information on some of the parasites prevalent among pre-20^{th} century Koreans. Throughout my investigation, I not only revealed the DNA sequences of those parasites but also elucidated, by phylogenetic analysis, their genetic characteristics. The obtained results show possible genetic differences in spatio-temporal aspects. In addition, the current report also establishes that the genetic characteristics of the parasite species were not uniform but rather were diverse to some degree.

In brief, in case of *T. trichiura*, the present study found a useful genetic marker for phylogenetic analysis. The analysis of *P. westermani* DNA also showed varying genetic characteristics according to geographic differences. Meanwhile, in the cases of *Ascaris* spp., *C. sinensis*, and *M. yokogawai*, I could not find clear evidence of intra-species genetic differences, though the final

determination in this matter will remain pending until a sufficient number of studies are performed.

Taking the present data together, and considering the very few reports on parasite aDNA that are available thus far, the current study significantly expands the existing genetic pool of the five parasite species examined. Nevertheless, I also admit that aDNA reports of a much wider geo-historical scope are still required in order to complete our knowledge of the exact evolutionary histories of these parasites. The present aDNA analysis on parasite eggs recovered from 15^{th} to 18^{th} century Joseon Dynasty mummy samples is, at least, a meaningful step in that direction.

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국문 초록

서론: 최근 분자생물학 연구의 발전은 옛 고대 전염병에 관한 연구에서 중요한 성과를 이루었지만 아직 옛 기생충 영역에서는 성과가 미흡하다. 지난 수년간 우리나라 고기생충학 연구를 통해 기생충란의 존재를 형태학적으로 확인한 옛 시료를 수습할 수 있었는데 이 연구에서는 기왕에 수집한 시료를 이용하여 회충, 편충, 폐흡충, 간흡충, 요코가와흡충 등 다양한 옛 DNA를 분석하고자 하였다.

연구 대상 및 방법: 15-18세기 조선시대 미라에서 수집한 장내 분변 혹은 조직 샘플을 연구에 이용하였다. 수집한 시료는 유전체 분석 전 현미경 검경을 통하여 각 시료 내 기생충란의 존재를 확인하였으며 고DNA를 추출한 다음 PCR로 증폭하여 서열을 확인하였다. 기존에 GenBank에 보고된 해당 기생충 서열과 유사도를 검색하는 한편, 이 서열에 대한 계통분석을 Maximum likelihood 법을 이용하여 실시하였다.

결과 및 고찰: 전기영동을 통해 조선시대 시료에서 추출한 회충, 편충, 폐흡충, 간흡충, 요코가와 흡충 등 DNA 증폭결과를 확인하였다. 회충 및 편충 DNA는 다른 기생충 DNA와 명확하게 구분되었으며 폐흡충 옛 DNA는 계통분석을 통해 지역에 따라 크게 세 그룹으로 나뉜다는 사실을 확인하였다. 간흡충 분석 결과 ITS1, NAD2, NAD5영역이 유전적 진단에 적합하다는 사실을 확인하였으며 요코가와흡충의 경우

CO1 영역에 대한 분석으로 해당 기생충을 분자생물학적으로 명확히 진단할 수 있다는 사실을 밝혔다. 이와 같은 연구를 통해 20세기 이전 동아시아 기생충의 유전적 특징을 밝히는 한편, 형태학적 특징만으로 진단이 어려운 고기생충란 연구에서 유전적 진단이 유용하게 쓰일 수 있다는 사실을 증명하였다.

결론: 이 연구에서는 우리나라 조선시대 미라에서 수집한 시료를 이용하여 회충, 편충, 폐흡충, 간흡충, 요코가와 흡충 고DNA 서열을 성공적으로 분석했다. 지금까지 기생충 옛 DNA에 대한 사례가 체계적으로 거의 분석되지 않았다는 점을 고려하면 이번 연구를 통해 획득한 기생충 서열 보고는 기존 고기생충학 유전자 풀을 확대하고 기생충의 정확한 진화사에 관한 정보를 얻는 데 있어 매우 중요한 자료가 될 수 있다.

중심어: 기생충; 회충; 편충; 폐흡충; 간흡충; 요코가와흡충; 고DNA

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감사의 글

박사학위를 받기 위해 노력했던 지난 3년간 많은 일들이 있었습니다. 수없이 많았던 좋았던 일, 힘들었던 일들을 뒤돌아보니 제가 여기까지 무사히 왔다는 사실이 놀라울 따름입니다. 늦은 나이에 공부하겠다고 나선 아들을 끝까지 믿고 기다려 주신 부모님, 박사과정 내내 곁에서 함께해 준 김선주 선생님, 힘들고 지칠 때마다 다시 일어날 수 있도록 큰 웃음을 준 친구들에게 감사를 드립니다.

지도교수님인 신동훈 교수님을 만나지 못했다면 이 연구를 수행할 수 없었을 것입니다. 배우는 것이 느린 학생이지만 지금까지 참을성 있게 저를 이끌어주신 지도교수님께 깊은 감사를 드립니다. 또한 제 논문 완성에 많은 조언을 해 주신 서울대학교 법의학교실 이숭덕 교수님, 기생충학교실 최민호 교수님, 해부학교실 최형진 교수님, 그리고 단국대학교 기생충학교실 서민 교수님께도 감사를 드립니다. 더불어 제가 학문적 소양을 갖출 수 있도록 조언을 아끼지 않으신 경희대학교 강인욱 교수님, 국립과학수사연구원 이원준 박사님, 단국대학교 김명주 교수님, 세종대학교 우은진 교수님께도 진심으로 감사드립니다.

제 연구는 수많은 선배 연구자들께서 장시간에 걸쳐 수집한 미라 샘플이 있었기에 소정의 성과를 거둘 수 있었습니다. 미라 샘플을 수집하는 데 큰 도움을 주신 오창석 선생님, 미라 부검에 도움을 주신 가톨릭대학교 김이석 교수님, 미라에서 수집한 분변샘플을 육안동정해주신

단국대학교 서민 교수님, 그리고 좀 더 나은 논문을 위해 조언을 해 주신 서울대학교 채종일 교수님께 감사드리며, 마지막으로 연구를 위해 샘플을 사용할 수 있도록 제공해 주신 각 문화재기관 연구자 분들께도 깊은 감사를 드립니다.

제 학위논문을 구성하는 5개의 Chapter 중 Chapter 1, 2는 각각 Journal of Parasitology (2016, 2019)에, Chapter 3은 Memórias do Instituto Oswaldo Cruz (2019), Chapter 4는 Journal of Korean Medical Science (2019) 등 학술지에 다른 공저자 분들과 함께 발표한 것을 본 학위논문 작성을 위해 다듬고 고쳐 완성하였음을 밝힙니다. 또한 Chapter 5의 내용은 정리하여 연내에 전문학술지에 투고할 계획입니다.

마지막으로 제게 도움을 주셨던 모든 분들께 다시 한번 진심으로 감사를 드립니다. 멈추지 않고 계속 노력하겠습니다. 감사합니다.